



# Developmental Maturation within the Hematopoietic System

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# **Developmental Maturation within the Hematopoietic System**

A dissertation presented

by

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to

**The Division of Medical Sciences**

In partial fulfillment of the requirements

for the degree of

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**Developmental and Regenerative Biology**

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# Developmental Maturation within the Hematopoietic System

## Abstract

Stem cell biologists creating cells and tissues for therapies, disease modeling, and drug screening have observed that differentiating pluripotent stem cells (PSCs) tend to produce cells at an embryonic stage of development but have difficulty maturing into adult definitive cells. A better understanding of developmental maturation will provide insights into embryogenesis and permit more accurate disease modeling. In the hematopoietic system, primitive and definitive cells are distinguished by functional transplantation assays, well characterized cell surface antigens, and gene expression signatures. We examined the transition *in vivo* in transplanted murine hematopoietic stem cells (HSCs) and *in vitro* in human PSC (hPSC) derived red blood cells (RBCs). We found that the hematopoietic microenvironment of the recipient significantly affects the outcome of HSC transplantation. The earliest embryonic HSCs perform better in neonatal recipients, whereas more mature adult-like HSCs perform better in adult recipients. The preference may be related to different active hematopoietic niches in neonates and adults, as we observed adult HSCs homing to different tissues in neonatal and adult recipients. Additionally, we found that proliferation may enhance the neonatal engraftment potential of adult-like HSCs. Our data highlight the importance of the host environment on transplantation outcomes, and point to the neonatal transplant model as a tool to functionally examine the earliest HSCs and primitive derivatives of PSCs.

We also investigated the maturation of RBCs derived from hPSCs and found that the RBC lineage derived *in vitro* is exclusively primitive. Exogenously expressing key transcription factors or adding stromal co-cultures during differentiation did not force the cells to mature to the definitive state, suggesting that culture conditions play a significant role and presently lack essential cues for the emergence of definitive hematopoietic cells. Additionally, the generated cells did not survive to contribute to the erythroid lineage when transplanted into a supportive *in vivo* environment. The system we optimized for differentiation of hPSCs into RBCs can be used to screen other factors to promote maturation to the definitive fate. Collectively, these data highlight the role of the microenvironment in determining the degree of developmental maturation of HSCs and their progeny.

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## **Statement of Contributions**

The work presented in Chapter 2 is under re-review at Developmental Cell. I am first author on the paper. Dr. Shannon McKinney-Freeman suggested the initial idea to examine early hematopoietic stem cell populations in the more permissive neonatal environment. Dr. McKinney-Freeman and I designed and performed transplants of E11.5 AGM into neonatal recipients. Dr. Pamela Wenzel and I designed and performed experiments with E9.5 PSp. I designed and performed all other experiments, analyzed and interpreted data, prepared the manuscript, and revised the manuscript along with Dr. George Daley. Sam Ross, a technician, assisted with transplants and figure creation. Sam Ross and Stephanie Chou, a technician, assisted with routine analysis of peripheral blood from transplant recipients. Dr. Mervin Yoder and Dr. Momoko Yoshimoto taught me how to perform intravenous neonatal injections. The Dana-Farber Cancer Institute Flow Cytometry Core assisted with cell sorting. Dr. Daley directed the project and provided conceptual framework.

Chapter 3 was published in part as Arora and Daley “Pluripotent stem cells in research and treatment of hemoglobinopathies” in Cold Spring Harbor Perspectives in Medicine. Dr. Daley and I conceptualized the work presented in Chapter 3. I designed and performed experiments as well as analyzed and interpreted data. Stephanie Chou assisted with experiments. The Children’s Hospital Boston Flow Cytometry Core assisted with cell sorting. Dr. Daley directed the project and provided conceptual framework.



*To my brother...*

*Let this inspire you to continue on your own journey through science.*

*And*

*To my husband...*

*Let us continue the pursuit of our intellectual passions together.*

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## **Chapter 1**

### **Introduction**

## 1.1 Primitive hematopoietic derivatives of pluripotent stem cells

For decades hematologic diseases, ranging from sickle cell anemia to leukemia, have been treated and/or cured by giving patients normal blood cells, which come from donated bone marrow, donated peripheral blood, or cord blood. Every year tens of thousands of patients in the United States depend on the availability of donor cells and a compatible donor cannot be found for 30% of patients [2]. Recent discoveries point to a potential alternative source of hematopoietic cells for therapy: human pluripotent stem cells (hPSCs). PSCs are derived from the inner cell mass of a blastocyst, called embryonic stem cells (ESCs), or somatic cells reprogrammed with exogenous expression of OCT4, SOX2, KLF4, cMYC, called induced pluripotent stem cells (iPSCs) [3-6]. hPSCs can be differentiated into many hematopoietic cells including hematopoietic stem cells (HSCs) and red blood cells (RBCs). Unfortunately, state of the art differentiation methods produce primitive cells that closely resemble the earliest hematopoietic cells in the embryo. In order to use hematopoietic derivatives of hPSCs for therapies, drug screening, or disease modeling, the cells need to be more mature, committed to the definitive fate as seen in hematopoietic cells in adults.

There is a major knowledge gap in the field in understanding the cell of origin and mechanism giving rise to definitive cells and how the mechanism differs from the emergence of primitive cells. There are also open questions surrounding the differences between primitive and definitive cells in response to the environment.

We made strides towards answering some of these questions in two systems: 1) maturation of embryonic HSCs or their precursors in murine neonates and 2) human induced pluripotent stem cells (hiPSCs) directly differentiated into RBCs *in vitro*. The murine hematopoietic system closely mimics the



human system, and provides an excellent model organism to functionally assay hematopoietic cells by transplantation.












## 1.2 Murine hematopoietic stem cell ontogeny




HSCs sit atop the hematopoietic hierarchy with the ability to self-renew and differentiate to generate all types of hematopoietic cells. HSCs are functionally defined by the ability to give rise to long-term multi-lineage reconstitution [7-10]. There is a debate amongst developmental hematologists as to when and where the first HSC arises in the murine embryo. Strong evidence supports the emergence of HSCs from the hemogenic endothelium of the para-aortic splanchnopleura/aorta-gonad-mesonephros (PSp/AGM) between embryonic day 9.0 (E9.0) and E11.5 [11, 12]. At E12.5, driven by an unknown mechanism, HSCs migrate to the fetal liver, where the stem cell pool expands [13-15]. The HSC niche in the fetal liver that promotes rapid proliferation has not been extensively studied. Beginning at E18.5, a loosely characterized mechanism prompts HSCs to seed the bone marrow, where they remain throughout the adult life of the mouse [16-20]. The prevailing hypothesis is that interaction with specific niches during development allows embryonic HSCs to mature into adult HSCs [21, 22]. Whereas the niches in the adult bone marrow that support HSC and progenitor function and maintenance are well studied and known to include CAR cells, mesenchymal stem cells, endothelial cells and osteolineage progenitors [23], the niches that support developmental maturation of HSCs are largely unstudied.

### 1.3 Developmental changes in murine hematopoietic stem cells

HSCs from different stages of development are not identical. HSCs emerging from the AGM tend to undergo more symmetric than asymmetric divisions to generate more HSCs. HSCs in the fetal liver are rapidly cycling to build up the stem cell pool for the organism, whereas HSCs in the adult bone marrow are mostly quiescent, largely allowing progenitors to maintain homeostasis. Also, the earliest HSCs do not have the same surface antigen profile as more mature HSCs, which suggests additional functional differences. E11.5 AGM HSCs can be prospectively isolated as VE-Cadherin<sup>+</sup> CD45<sup>+</sup> CD34<sup>-</sup> cKit<sup>low</sup> Sca1<sup>+</sup> CD31<sup>low</sup>, while a nearly pure population of fetal liver and adult bone marrow HSCs is Lineage<sup>-</sup> Sca1<sup>+</sup> cKit<sup>low</sup> CD150<sup>+</sup> CD48<sup>-</sup> [24-27].

Table 1-1: Early Embryonic HSCs in Different Transplant Models

Donor Age	Wild-type Adult Recipient	Immunodeficient Adult Recipient	Wild-type Neonatal Recipient	Imaging
E11.5 AGM				
E10.5 AGM				
E10.0 YS				
E9.5 PSp				
E9.0 YS				

 engraftment     
  no engraftment     
  rare engraftment

The earliest HSCs also have different engraftment potential in various transplant models (Table 1-1). In lethally irradiated wild-type adult mice, the current gold standard functional assay, limiting dilution transplants predicted 1 HSC in the AGM of each E11.5 embryo. E10.5 AGM rarely engrafts wild-type adult mice, and no engraftment from E9.5 PSp has been reported in wild-type adult mice [19, 28, 29]. However, if the host environment is altered by transplanting into immunodeficient adult mice, then E10.5 AGM and even E9.5 PSp can engraft [30]. These early HSCs can also engraft a second model system: wild-type neonatal recipients [31]. Cells from E9.5 PSp and even E9.0 yolk sac give long-term multi-lineage engraftment in wild-type neonates and can reconstitute secondary recipients [32-35]. These different transplant models can be used to further our understanding of the earliest HSCs, how they emerge, what signals and environment they need to survive and mature, and how they differ from more mature HSCs.

Within engrafting adult-like HSCs, there is heterogeneity in terms of contribution to myeloid and lymphoid lineages. Eaves and colleagues identified 4 types of HSCs in fetal liver and adult bone marrow based on relative lineage contributions [36, 37]. While all 4 types are detectable in fetal liver,  $\beta$ -HSCs are dominant and  $\alpha$ -HSCs are rare. Once the bone marrow is established at E18.5, the number of  $\alpha$ -HSCs increases until it is half of the  $\beta$ -HSCs. This ratio is present in the bone marrow of 3-week-old mice and remains in adult bone marrow, suggesting the bone marrow stimulates the increase in  $\alpha$ -HSCs.

#### 1.4 Developmental changes in lineage committed hematopoietic cells

Like HSCs, mature lineage committed hematopoietic cells have characteristics unique to embryonic and adult hematopoiesis. In B-cell lymphopoiesis, B-1a cells are seen in transplants of

neonatal liver, whereas B-2 cells are observed in transplants of both neonatal liver and adult bone marrow [38]. Splenic marginal zone B-cells have a fetal origin [39, 40]. In T-cell lymphopoiesis, fetal T-cells express the V $\gamma$ 3 V $\delta$ 1 or V $\gamma$ 4 V $\delta$ 1 T-cell receptors, while adult T-cells express V $\gamma$ 2 or V $\gamma$ 5 [41, 42]. Fetal monocytes have increased expression of tissue-degrading enzymes, scavenger receptors, and chemokines and, unlike adult cells, can transition from CD14<sup>low</sup> CD16<sup>-</sup> precursors to CD14<sup>high</sup> CD16<sup>+</sup> without passing through a CD14<sup>high</sup> CD16<sup>-</sup> intermediate [43]. Fetal and adult megakaryocytes undergo a similar process to produce platelets, but fetal megakaryocytes release platelets when the cells have lower ploidy and are smaller [44-47]. Erythrocytes produce different hemoglobins during embryogenesis and in adulthood, which will be described in detail in sections 1.5 and 1.6.

### 1.5 Human erythropoiesis

Erythropoiesis occurs in two waves: primitive and definitive. Large nucleated primitive erythroblasts containing  $\epsilon$ -globin arise in the yolk sac and provide the embryo with RBCs until definitive cells residing in the fetal liver and begin producing fetal definitive RBCs containing  $\gamma$ -globin. This constitutes the first globin switching event in humans. A second globin switching event occurs as the bone marrow becomes the main site of definitive hematopoiesis and results in RBCs containing  $\beta$ -globin [48]. Other than hemoglobin expression and size, primitive and definitive RBCs also differ by i/I expression, expression of  $\delta$ -globin, carbonic anhydrase content, and the level of activity and function of phosphofructokinase [49].

HSCs differentiate into many mature cell types including enucleated RBCs. Differentiation into RBCs goes through six identifiable stages during which hemoglobin protein is accumulated and the

nucleus compacts to eventually be extruded from the cell leaving a 6-8  $\mu\text{m}$  mature RBC [50]. Cell surface antigens Band 3 and  $\alpha 4$ -integrin can be used to identify and purify cells at each stage [51].

## 1.6 Globin switching

The  $\beta$ -globin locus, positioned on human chromosome 16, contains five different hemoglobin genes arranged in the order they are expressed during development. The mechanisms regulating gene expression at the  $\beta$ -globin locus are not completely understood. The looping model of globin regulation proposes that the distal locus control region (LCR) loops around to bring activator proteins in proximity to proteins at the promoter of each globin gene for transcriptional activation [52-55]. A dual mechanism combining autonomous gene control and gene competition for interaction with the LCR upstream of the globin locus has been proposed to explain globin switching [56, 57]. When only proximal *cis*- regulatory elements are present, the globin genes are expressed at the correct developmental stage and in the proper tissue but in a position dependent fashion [58-61]. These observations suggest autonomous gene control. However, that is not the only mechanism in action. It has been proposed that at the time of the second globin switch from  $\gamma$ -globin to  $\beta$ -globin, a flip-flop mechanism allows a dynamic expression of both  $\gamma$ -globin and  $\beta$ -globin until repressive factors autonomously silence  $\gamma$ -globin expression [62]. Then,  $\beta$ -globin expression increases, and it remains the predominant globin throughout adulthood. This suggests a possible competition between the two globin genes for interaction with the LCR. Many transcription factors and complexes including BCL11A, KLF1, SOX6, GATA1, NF-E4, COUP-TF, IKAROS, DRED, and MBD2 are known to bind the  $\beta$ -globin locus, but the exact mechanism regulating globin expression is unknown [63-75].

## 1.7 Directed differentiation of hPSCs cells to RBCs

Before using human PSCs for cell therapy, it must first be possible to differentiate human PSCs into the desired cell type. Many groups have attempted to recapitulate *in vivo* erythropoiesis by differentiating hESCs into RBCs *in vitro* (Table 1-2) [1]. Directly differentiating human PSCs into definitive RBCs has proven challenging. It is possible to generate large numbers of primitive immature RBCs, but generating definitive adult-like RBCs has proven elusive for the field. The cells accumulate hemoglobin, appear red in color, and bind oxygen. They mature appropriately as measured by cell surface markers CD71 and GPA, but remain mostly nucleated and have a primitive hemoglobin expression pattern, expressing  $\epsilon$ -globin and  $\gamma$ -globin with little or no  $\beta$ -globin. The system from Lu *et al.* is the only one with serum free conditions without co-culture. They generate few erythroid cells that have the capacity to express low levels of  $\beta$ -globin but still express high levels of  $\gamma$ -globin and  $\epsilon$ -globin [76]. MacLean *et al.* comes the closest to generating mature definitive RBCs. They generate cells producing mostly  $\gamma$ -globin, but the RBC yield is low. An unanswered question in the field is what mechanism will drive the switch from primitive to definitive RBCs *in vitro*.

Table 1-2: Differentiation of hPSCs to RBCs

<sup>a</sup>Number of erythroid cells generated per hESCs or hiPSCs. Hb: hemoglobin tetramer; N/M: not mentioned; Enu: enucleation; N/A: not applicable; CB: cord blood; PB: peripheral blood; BL: hemangioblasts; MSC: mesenchymal stem cells. Figure modified from Chang *et al.* [1].

Reference	Stem cell lines	Method	Stromal cells	Major globin chains	Hb	Enu (%)	Yield <sup>a</sup>	Culture length (days)	Note
Macleod <i>et al.</i> [77]	CSE2, CSE13, DS2-iPS1, DS2-iPS10, DS1-iPS4, MRC5-iPS7	EB	N/A	$\gamma$	N/M	N/M	N/M	10	
Dias <i>et al.</i> [78]	H1, hiPSCs	coculture	OP9 → MS5	$\epsilon, \gamma$	N/M	2–10	200,000	75	
Kaufman <i>et al.</i> [79]	H1	coculture	S17, C166, MEF	$\alpha, \beta, \delta$	N/M	N/M	N/M	42	
Klimchenko <i>et al.</i> [80]	H1, H9	coculture	OP9 → MS5	$\zeta, \epsilon$	N/M	no	N/M	38	
Lee <i>et al.</i> [81]	H1, H9, H14	coculture	AGM, FL, FBM	$\alpha, \gamma, \delta$	N/M	no	N/M	32	
Ledran <i>et al.</i> [82]	H1, H9, hES-NCL1	coculture	AGM, FL	$\alpha, \epsilon, \gamma$	N/M	N/M	N/M	39	

Table 1-2: Differentiation of hPSCs to RBCs (Continued)

Maet <i>et al.</i> [83]	H1	coculture	mFLSC	$\epsilon, \gamma, \beta$	N/M	6.2	1,200	34	Time-dependent $\beta$ globin increase at clonal level. Oxygen dissociation curve and G6PD activities are more similar to CB than to adult PB
Olivier <i>et al.</i> , Qiu <i>et al.</i> [84-86]	H1	coculture	FH-B-hTERT, S17	$\zeta, \alpha, \epsilon, \gamma$	N/M	1.5 - 1.6	80	59	Maturation switch and lineage switch noted. Minor increase in $\beta$ globin expression
Cerdan <i>et al.</i> [87]	H1, H9	EB	N/A	$\epsilon, \beta$	HbA > HbF	N/M	N/M	29	
Chang <i>et al.</i> [88-90]	H1, BG02, hiPSCs	EB	N/A	$\alpha, \epsilon, \gamma$	N/M	N/M	N/M	21	
Lapillonne <i>et al.</i> [91]	H1, hiPSCs	EB	N/A	$\alpha, \gamma$	HbF	4-66	200-3,500	46	CO rebinding kinetics similar to CB



Table 1-2: Differentiation of hPSCs to RBCs (Continued)

Zambidis <i>et al.</i> [92]	H1	EB	N/A	$\zeta, \alpha, \epsilon, \gamma$	HbF > HbA	N/M	N/M	36	Time-dependent increase in $\beta$ globin expression observed. Primitive to definitive switch noted
Lu <i>et al.</i> [76]	H1, MA01, MA99, HuES-3	EB $\rightarrow$ BL	MSC, OP9	$\zeta, \alpha, \epsilon, \gamma$	N/M	10–30; 30–65	800– 8,000	25	Oxygen dissociation curve slightly displaced to left of normal RBCs. Decreased response to 2,3-DPG depletion as compared to adult RBCs. Comparable Bohr effect at physiologic pH

Table 1-2: Differentiation of hPSCs to RBCs (Continued)

Honig <i>et al.</i> [93]	H1, H7, MA01	EB → BL	N/A		Hb Gower I > Hb Barts	N/M	N/M	25	Low $(\alpha + \zeta)/(\gamma + \epsilon)$ synthesis ratio
Liu <i>et al.</i> [94]	H1	EB → coculture	hFLSC	$\zeta, \alpha, \epsilon, \gamma$	N/M	N/M	N/M	29	$\beta$ globin expression increases with culture time. Oxygen dissociation curve slightly displaced to left of normal RBCs

This thesis highlights the affect of the environment on the ability of hematopoietic cells to mature to the adult definitive fate. In Chapter 2 we test the hypothesis that neonates are more permissive hosts for hematopoietic stem cell transplants and provide insights to the mechanism governing the preference of HSCs for different hosts. In Chapter 3 we examined the potential of human pluripotent stem cell derived RBCs to mature to definitive RBCs.

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## **Chapter 2**

### **Neonatal engraftment defines nascent embryonic HSCs**

## 2.1 Abstract

The first hematopoietic stem cells (HSC) that engraft irradiated adult mice arise in the aorta-gonad-mesonephros (AGM) on embryonic day 11.5 (E11.5). However, at this stage there is a discrepancy between the apparent frequency of HSCs suggested by imaging and their rarity when measured by limiting dilution transplant. We have attempted to reconcile this difference using neonatal recipients, which are more permissive for embryonic HSC engraftment. We found that embryonic HSCs from E9.5 and E10.5 preferentially engrafted neonates, whereas developmentally mature, definitive HSCs from E14.5 fetal liver or adult bone marrow more robustly engrafted adults. Neonatal engraftment was enhanced after treating adult bone marrow-derived HSCs with interferon. Adult bone marrow-derived HSCs preferentially homed to the liver in neonatal mice yet showed balanced homing to the liver and spleen in adults. As more receptive hosts, neonates enable detection and quantification of emerging definitive HSCs as early as E9.5.

## 2.2 Introduction

According to the classical definition, a definitive HSC reconstitutes multi-lineage hematopoiesis long-term in irradiated adult primary transplant recipients, and can be serially passed into secondary recipients, indicating the capacity for both self-renewal and multi-lineage differentiation [1-4]. Under that definition, the first transplantable murine HSC emerges *in vivo* in the AGM at E11.5 [5-9]. HSCs then migrate to the fetal liver and rapidly divide to build up the stem cell pool. Ultimately, HSCs populate the developing bone marrow where they become quiescent once homeostasis is reached in the post-natal

period. Interaction with specific niches during development allows embryonic HSCs to mature into adult HSCs [10, 11].

The surface antigen profile of the engrafting cell at E11.5, as identified by transplantation, is VE-Cadherin<sup>+</sup> CD45<sup>+</sup> CD34<sup>-</sup> cKit<sup>low</sup> Sca1<sup>+</sup> CD31<sup>low</sup> [12, 13]. Imaging techniques coupled with the cell surface profile of the engrafting cell have provided insight into the emergence of the definitive HSC [14-17]. Imaging has revealed hundreds of c-Kit<sup>+</sup> clusters in E11.5 AGM, implying a discrepancy in the quantity of potential HSCs identified by imaging relative to the frequency defined by limiting dilution transplantation in adult recipients.

Analysis of emerging hematopoietic cells in the AGM at E10.5 shows 1.7 Sca1<sup>+</sup> c-Kit<sup>+</sup> CD31<sup>+</sup> CD41<sup>+</sup> cells per embryo [16]. Transplantation has revealed that E10.5 AGM cells are capable of rare long-term multi-lineage repopulation of wild-type adult recipients (3% of transplanted animals) and more robust repopulation in immunodeficient adult recipients, indicating that the host environment plays a critical role in detection of nascent HSCs [8, 18, 19]. Interestingly, VE-Cadherin<sup>+</sup> CD45<sup>-</sup> cells from E10.5 AGM robustly reconstitute wild-type adult recipients after 4 days of *ex vivo* culture, further suggesting that there are cells in the AGM at E10.5 with enhanced potential to engraft if cultured under proper conditions [20].

Before E10.5, the tissues of the para-aortic splanchnopleura (PSP) contain progenitors that are primed to give rise to the definitive HSC, as per the classical definition [8, 21, 22]. A population from E9.5 PSP has been reported to engraft immunodeficient adult recipients and wild-type neonatal recipients [23-25]. Neonatal engraftment has also been observed from E9.0 yolk sac [26, 27], suggesting that the neonate may be more permissive for engraftment of early embryonic HSCs. In this study we utilized the neonatal transplant model to quantify nascent definitive HSCs in the earliest intraembryonic tissues to date, and confirm the more permissive environment of the neonatal recipient. Interestingly,

we also found that definitive adult HSCs engraft less robustly in the neonate relative to the adult. Our data demonstrate the significant impact of the recipient on the outcome of HSC transplantation, and define distinct survival and engraftment requirements for adult HSCs.

## 2.3 Results

We first compared engraftment of neonates and adults with cells from E11.5 AGM, the earliest population reported to engraft wild-type adult recipients. Neonatal recipients proved to be more permissive for engraftment than adult recipients with similar conditioning. Whereas 7/9 neonatal recipients were engrafted with 1ee, at most 3/6 adult recipients receiving sub-lethal irradiation and no helper cells were engrafted at low levels (Figure 2-1 A and Table 2-1). Neonates transplanted with E11.5 AGM and helper cells were engrafted at levels similar to those transplanted without helper cells, indicating that the helper cells are not responsible for the low level engraftment observed in adult recipients (Figure 2-2). Three of 11 neonates transplanted with 0.25 embryo equivalents (ee) showed long-term engraftment (Figure 2-3 A and B). Secondary transplants demonstrated donor HSC self-renewal when taken from primary animals with multi-lineage reconstitution (Figure 2-3 C), but three primary recipients with long-lived myeloid biased lineage skewing did not engraft secondary recipients, and thus were not engrafted with a self-renewing HSC (Figure 2-3 B). We also compared neonatal engraftment with E11.5 AGM to the current gold standard of lethally irradiated adult recipients receiving helper cells. Only 1/8 adult mice was engrafted (Table 2-1). Before quantitative analysis, these data suggest the neonatal transplant assay is a more permissive functional test for early embryonic HSCs, and the preference for neonatal recipients is even more marked when compared to the gold standard assay.

Figure 2-1

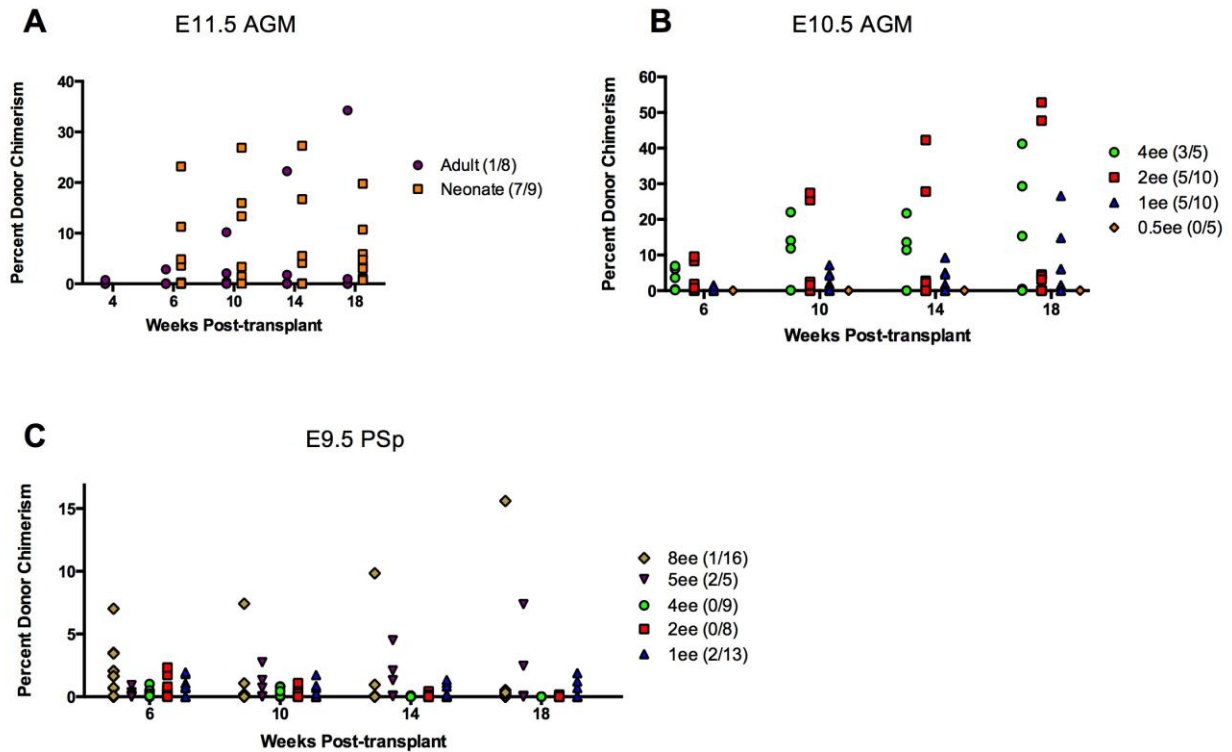


Figure 2-1: Neonates are more permissive for engraftment of early embryonic HSCs. A) Adult (circle) and neonatal (square) recipients were both transplanted with 1ee of whole E11.5 AGM. Neonatal recipients were transplanted with limiting doses of B) whole E10.5 AGM and C) whole E9.5 PSp. The numbers in the graph legends reflect the number of animals engrafted over the number of animals transplanted.

Figure 2-2

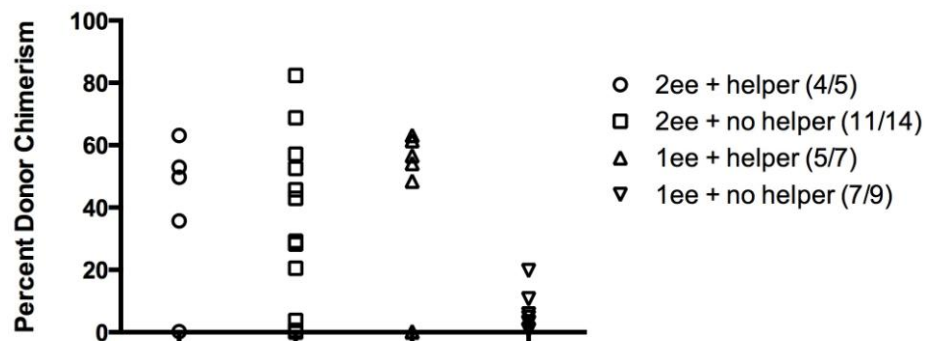


Figure 2-2: Helper cells in neonatal transplant model. Neonates received 2ee or 1ee of E11.5 AGM along with 500,000 whole bone marrow cells as helper. Data shown are 18 weeks post-transplant.

Figure 2-3

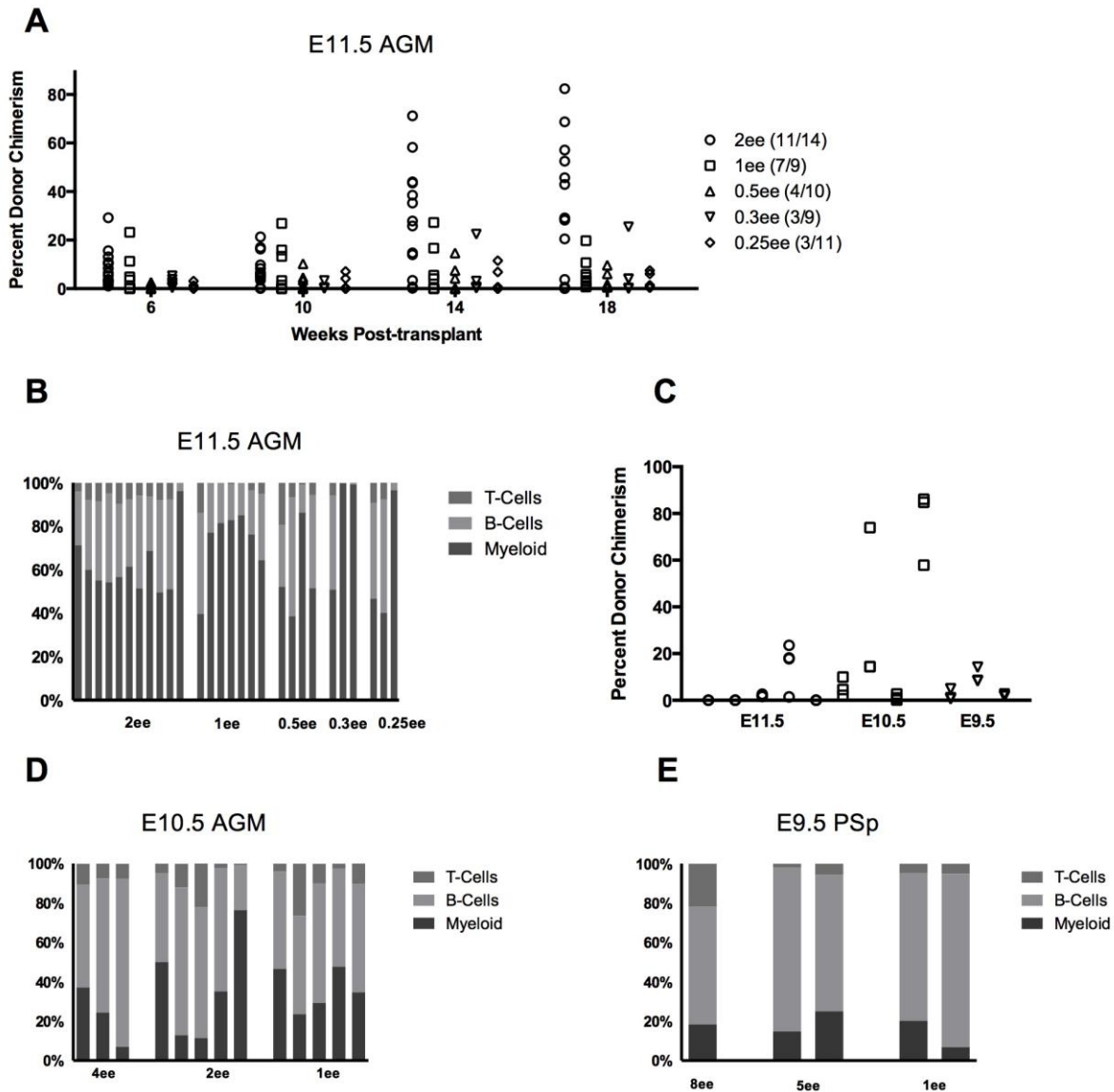


Figure 2-3: Multi-lineage reconstitution and self-renewal capacity of early embryonic HSCs. A) Neonatal recipients were transplanted with limiting doses of whole E11.5 AGM. The numbers in the graph legend reflect the number of animals engrafted over the number of animals transplanted. B) Lineage breakdown of donor contribution in peripheral blood at 18 weeks post-transplantation for neonates engrafted with E11.5 AGM. C) Secondary transplants were carried out for primary recipients engrafted



Figure 2-3 (Continued)

with early embryonic HSCs. Lineage breakdown of donor contribution in peripheral blood at 18 weeks post-transplantation for neonates engrafted with D) E10.5 AGM and E) E9.5 PSp.

Table 2-1: Neonatal engraftment from early embryonic HSCs. The threshold for engraftment was drawn at 1%. Percent engraftment was calculated using only engrafted mice. P-values are between neonatal and adult recipients transplanted with the same donor population and calculated using Fisher's exact test. ND – not determined.

Donor		Recipient	Irradiation Dose (Gy)	Number of Animals Engrafted	Number of Animals Transplanted	Percent Animals Engrafted	Average Percent Donor Chimerism	p-value
E11.5	1 ee	Adult	3.5	2	6	33.3	3.4	ND
	1 ee	Adult	6.5	3	6	50	2.1	ND
	1 ee	Adult	10	1	8	12.5	34.2	0.015
	1 ee	Neonate	3.5	7	9	77.8	6.9	
E10.5	1 – 7 ee	Adult	10	0	20	0	0	0.0018
	1 ee	Neonate	3.5	5	10	50	11	
E9.5		Adult	10	ND	ND	ND	ND	ND
	1 – 8 ee	Neonate	3.5	5	53	9.4	5.7	

Figure 2-4

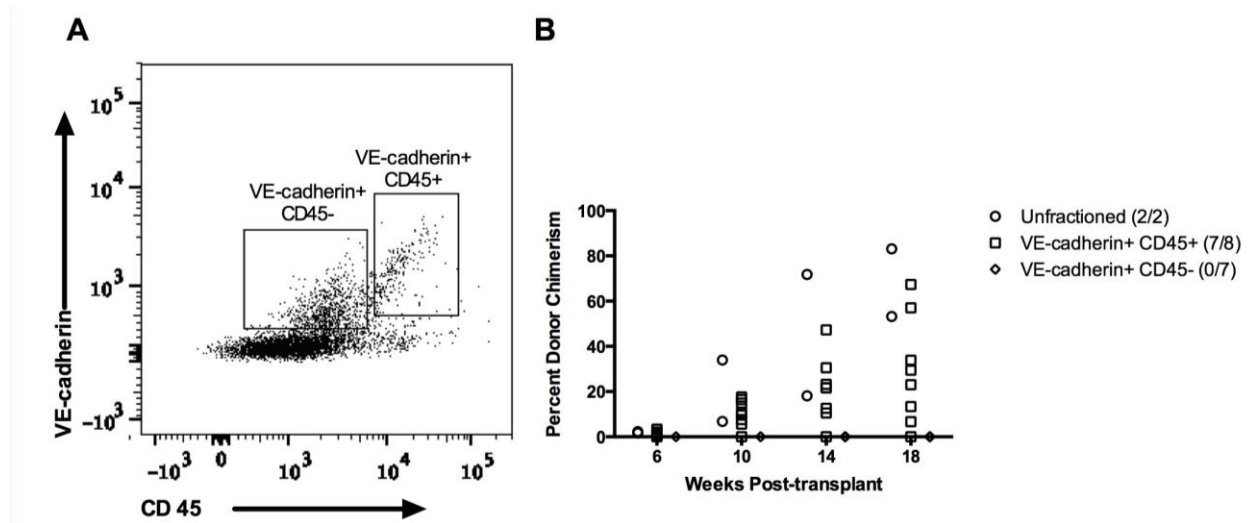


Figure 2-4: Adult engrafting subpopulations also engraft neonatal recipients. A) FACS plot of E11.5 AGM fractionated by VE-Cadherin and CD45. B) Neonatal recipients transplanted with unfractionated, VE-Cadherin<sup>+</sup> CD45<sup>+</sup>, or VE-Cadherin<sup>+</sup> CD45<sup>-</sup> cells from E11.5 AGM. The numbers in the graph legend reflect the number of animals engrafted over the number of animals transplanted.

VE-Cadherin<sup>+</sup> CD45<sup>+</sup> cells from E11.5 AGM have been reported to engraft adult recipients [13]. Similarly, we found that the VE-Cadherin<sup>+</sup> CD45<sup>+</sup> but not the VE-Cadherin<sup>+</sup> CD45<sup>-</sup> compartment engrafted neonates, thus demonstrating that neonates and adults are engrafted with the same cells from E11.5 AGM (Figure 2-4 B). However, the predicted repopulating cell frequency was higher in neonatal than adult recipients. Using the neonatal transplant model, limiting dilution analysis (LDA) predicted 1 repopulating cell in 0.935 ee, thus quantifying the sensitivity of the neonatal system for engraftment of embryonic HSCs (Table 2-2). In our hands using the adult transplant model with 300,000 helper cells, LDA predicted 1 repopulating cell in 5.7 ee of E11.5 AGM, whereas the published frequency using adult recipients is approximately 1 per ee [5] (Table 2-2). When we replicated the conditions from

Kumaravelu *et al.* using 20,000 helper cells, the calculated frequency was 1 repopulating cell in 0.538 ee (Table 2-2). Thus, our experimental quantifications reveal sensitivity in the adult transplant model to helper dose and no preference of HSCs at E11.5 for neonatal recipients.

Table 2-2: Early embryonic but not adult-like HSCs prefer neonatal recipients. LDA of reconstitution of neonatal and adult recipients with adult bone marrow LT-HSCs, E14.5 fetal liver LT-HSCs, whole E11.5 AGM, whole E10.5 AGM, and whole E9.5 PSp. The upper and lower limits provide the 95% confidence interval for the repopulating cell frequency. The goodness of fit tests how well the data fit the single-hit model that one repopulating cell will give a positive signal. A value of 1 suggests a good fit, less than 1 suggests heterogeneity in the donor cell population, and greater than 1 suggests a multi-hit model where recipients are hypersensitive to the given dose of donor cells. P-values are between neonatal and adult recipients transplanted with the same donor population and were calculated by the LDA algorithm. ND – not determined.

Donor	Recipient	Helper Cell Dose	Estimated Repopulating Cell Frequency	Frequency Upper Limit	Frequency Lower Limit	Fit Single-hit Model	p-value
BM LT-HSCs	Adult	300,000	1/13.1 cells	1/6.19	1/27.7	0.96	3.33E-8
	Neonate	None	1/177 cells	1/100	1/313	1.2	
FL LT-HSCs	Adult	300,000	1/25.7 cells	1/13.8	1/47.6	0.55	3.07E-4
	Neonate	None	1/114 cells	1/66.9	1/195	0.91	
E11.5 AGM	Adult	20,000	1/0.538 ee	1/0.24	1/1.2	-4.46	5.12E-5
		300,000	1/5.7 ee	1/2.16	1/15	2.6	
	Neonate	None	1/0.935 ee	1/0.624	1/1.4	0.74	
E10.5 AGM	Adult		ND	ND	ND	ND	ND
	Neonate	None	1/2.84 ee	1/1.6	1/5.02	0.71	
E9.5 PSp	Adult		ND	ND	ND	ND	ND
	Neonate	None	1/44.8 ee	1/18.4	1/109	-0.212	

We then evaluated the engraftment potential of newly specified HSCs from earlier stages of ontogeny. Neonates showed robust, long-term, multi-lineage reconstitution from 4 ee to 1 ee of unfractionated E10.5 AGM (30-39 somites) (Figure 2-1 B and Figure 2-3 D). At 18 weeks post-transplant, 3/5 recipients receiving 4 ee, 5/10 receiving 2 ee, and 5/10 receiving 1 ee showed multi-lineage engraftment with an average of 28%, 22%, and 11% donor contribution, respectively. No neonates were engrafted with 0.5 ee. In comparison, no engraftment was observed in adult recipients receiving up to 7 ee with as few as 20,000 helper cells (Table 2-1). Secondary transplants demonstrated self-renewal capacity of the repopulating donor cells from E10.5 AGM (Figure 2-3 C). LDA predicted 1 repopulating cell in 2.84 ee of E10.5 AGM when using the neonatal transplant model (Table 2-2), indicating that the repopulating cell is less abundant in the AGM at E10.5 than it is at E11.5. These data establish that whole E10.5 AGM robustly reconstitutes neonatal but not adult recipients, and demonstrate the existence of an HSC capable of long-term multi-lineage reconstitution in the AGM of some embryos as early as E10.5.

When we examined reconstitution potential from even earlier in ontogeny, we detected long-term multi-lineage donor chimerism from unfractionated E9.5 PSp (16-26 somites) transplanted into neonates in doses from 8 ee to 1 ee (Figure 2-1 C and Figure 2-3 E). At 18 weeks post-transplant, 1/16 recipients receiving 8 ee, 2/5 receiving 5 ee, and 2/13 receiving 1 ee were engrafted at an average of 15%, 5%, and 2%, respectively. Curiously, mice engrafted with E9.5 PSp showed an early B-cell skewing, which was not as pronounced in neonates engrafted with E10.5 AGM and not seen with E11.5 AGM (Figure 2-3 E). LDA predicted 1 repopulating cell in 44.8 ee of E9.5 PSp (Table 2-2). Secondary transplants confirmed that the engrafting cell from E9.5 PSp was truly a self-renewing stem cell (Figure 2-2 C). These findings provide additional conclusive evidence for the existence of an intraembryonic repopulating cell as early as E9.5 in some embryos.

We next determined if the neonate is a more permissive recipient for all HSCs or only those from the early embryo. LT-HSCs (Lineage<sup>-</sup> Sca1<sup>+</sup> c-Kit<sup>+</sup> CD150<sup>+</sup> CD48<sup>-</sup>) from adult bone marrow [28] were transplanted into neonatal and adult recipients in doses ranging from 100 cells to 10 cells (Figure 2-5 A and Table 2-3). Surprisingly, adult donor LT-HSCs failed to give rise to robust engraftment in neonatal recipients monitored for up to 23 weeks. Adult recipients were robustly engrafted, whereas engraftment in neonatal recipients was minimal. At 18 weeks post-transplant, all adult recipients that received 100 cells and 3 of 5 animals that received 10 cells showed multi-lineage engraftment. LDA from adult recipients predicted 1 repopulating cell in 13.1 LT-HSCs from adult bone marrow, which is less frequent than published reports, which used 200,000 helper cells [28]. While considering low-level engraftment of neonates, LDA predicted 1 repopulating cell in 177 LT-HSCs (Table 2-2).

We mined published microarray data of HSCs from E11.5 AGM and adult bone marrow to better understand why HSCs from different developmental stages might prefer different transplant hosts [29]. Modules from the microarray data point to key differences in cytokine-cytokine receptor interaction, transcription, metabolism, the toll-like receptor signaling pathway, and membrane composition. With 324 differentially expressed genes encoding membrane proteins, we hypothesized HSCs from various points in ontogeny may home to unique niches in different hematopoietic tissues. Interestingly, all examined genes known to be involved in homing (cxcr4, cd44, connexin-43, cd49d, lfa-1, and psgl1) showed consistent expression between E11.5 AGM HSCs and adult bone marrow HSCs.

Figure 2-5

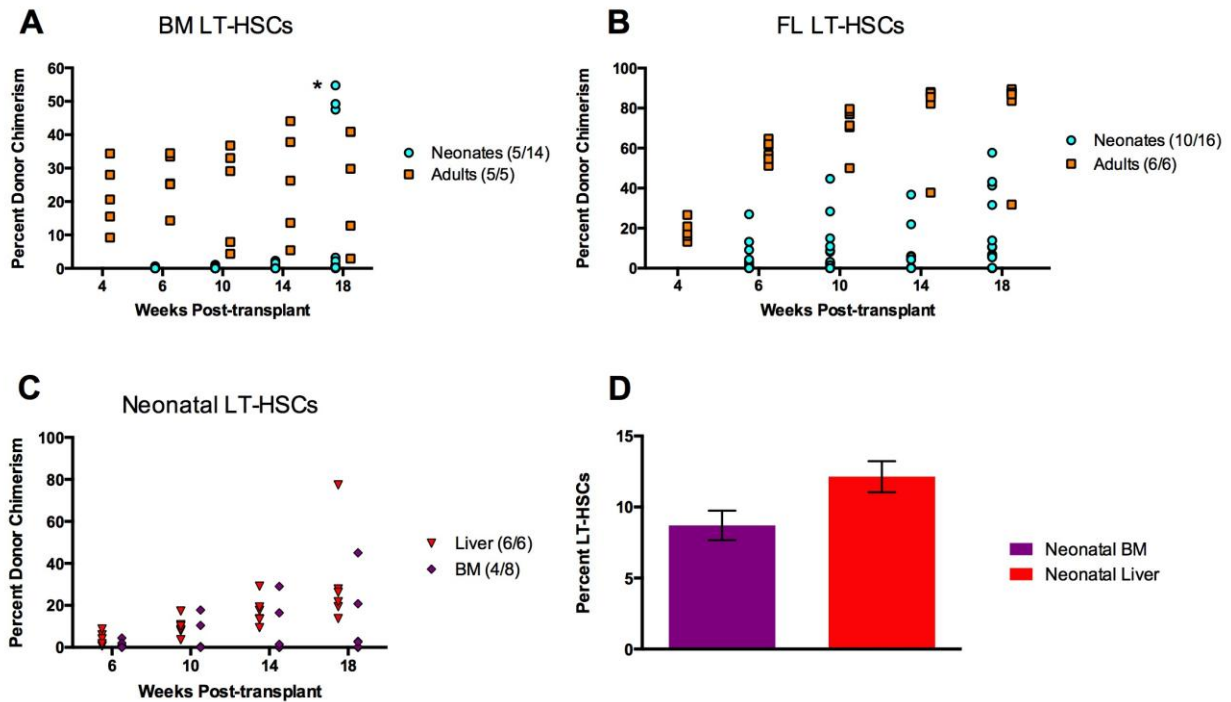


Figure 2-5: Neonatal recipients are not permissive for engraftment of all HSCs. A) Neonatal (circles) and adult (squares) recipients were transplanted with 100 adult bone marrow LT-HSCs. \*While in 3 neonatal recipients we detected a robust signal in the peripheral blood at a single time point of 18 weeks in one experiment, substantially lower donor chimerism was observed in these mice at the next time point of 23 weeks, and no other experiments showed this phenomenon, leading us to conclude that these data may have been a spurious experimental artifact. B) Neonatal (circles) and adult (squares) recipients were transplanted with 100 E14.5 fetal liver LT-HSCs. C) Neonatal recipients were transplanted with 50 neonatal liver LT-HSCs (circles) and 50 neonatal bone marrow LT-HSCs (squares). The numbers in the graph legends reflect the number of animals engrafted over the number of animals transplanted. D) Quantification of FACS analysis of Ki-67<sup>+</sup> LT-HSCs in neonatal liver and bone marrow. Data shown is the mean  $\pm$  SEM of three experimental replicates with pooled samples.

Table 2-3: Neonatal engraftment from adult-like HSCs. The threshold for engraftment was drawn at 1%. Percent engraftment was calculated using only engrafted mice. P-values are between neonatal and adult recipients transplanted with the same donor population. Statistical analysis for adult BM HSCs was performed using Fisher's exact test, and the student's t-test was used for fetal liver HSCs and neonatal HSCs. \*3/5 animals had robust engraftment that was not seen at any other time point up to 23 weeks post-transplant leading us to conclude it may have been a spurious experimental artifact. ND – not determined.

Donor		Recipient	Number of Animals Engrafted	Number of Animals Transplanted	Percent Animals Engrafted	Average Percent Donor Chimerism	p-value
Adult BM	100 Cells	Adult	5	5	100	25.4	0.033
	100 Cells	Neonate	5*	14	35.7	31.4	
E14.5 FL	100 Cells	Adult	6	6	100	77.9	1.44E-6
	100 Cells	Neonate	10	16	62.5	22.7	
Neonatal BM	50 Cells	Neonate	4	8	50	17.8	0.058
Neonatal Liver	50 Cells	Neonate	6	6	100	31.0	

To determine if a mature definitive HSC population from the fetal liver could engraft neonates, we transplanted cells with the same surface antigen profile (Lineage<sup>-</sup> Sca1<sup>+</sup> c-Kit<sup>+</sup> CD150<sup>+</sup> CD48<sup>-</sup>) from E14.5 fetal liver into neonatal and adult recipients in doses ranging from 100 cells to 10 cells (Figure 2-5 B and Table 2-3). Adult recipients had high level engraftment at all doses, whereas neonatal engraftment was only robust with 100 donor cells. At 18 weeks post-transplant, 10/16 neonatal recipients that received 100 cells showed balanced multi-lineage reconstitution with an average of

22.7% donor contribution; whereas only 1/8 neonates transplanted with 50 cells, 2/6 transplanted with 25 cells, and 1/8 transplanted with 10 cells were engrafted. LDA from neonatal recipients predicted 1 repopulating cell in 114 LT-HSCs; whereas from adult recipients LDA predicted 1 repopulating cell in 25.7 LT-HSCs (Table 2-2). Thus, while E14.5 fetal liver LT-HSCs engrafted neonates more readily than adult bone marrow LT-HSCs, both classes of definitive LT-HSCs from fetal liver and adult bone marrow engrafted better in irradiated adults than in neonates.

To further examine the differences between HSCs residing in the liver versus the bone marrow, we transplanted LT-HSCs from neonatal bone marrow and neonatal liver into neonates. At 18 weeks post-transplant, we observed a difference in the engraftment potential; more animals were engrafted at higher levels with neonatal liver LT-HSCs (Figures 2-5 C and Table 2-3). More neonatal liver LT-HSCs are proliferating than neonatal bone marrow LT-HSCs (Figure 2-5 D). These data show neonatal engraftment potential as well as proliferation decreases around birth in HSCs that have migrated to the bone marrow.

Fetal liver LT-HSCs are rapidly cycling and expanding in the developing embryo, while adult bone marrow LT-HSCs are typically quiescent [30-33]. Thus we tested whether proliferation status could influence the potential for definitive adult-like HSCs to engraft neonates. To drive adult-like HSCs into the cell cycle, we treated adult mice with interferon- $\alpha$  (IFN $\alpha$ ) and 5-FU [34-36]. Cycling HSCs were then isolated and transplanted into neonatal recipients (Figure 2-6 A). HSCs exposed to IFN $\alpha$  were able to engraft neonates and adults more robustly than PBS treated controls (Figure 2-6 B). When HSCs were exposed to 5-FU, which has less effect on quiescent HSCs than IFN $\alpha$  [37], we observed no enhancement of neonatal engraftment (Figure 2-6 C). Further analysis of HSCs treated with IFN $\alpha$  and 5-FU through published microarrays revealed a multitude of differentially expressed genes, suggesting IFN $\alpha$  and 5-FU have very different effects on HSCs, which may explain the difference in neonatal engraftment potential



[34, 38]. These data, in addition to the reduction of cycling LT-HSCs in neonatal bone marrow, suggest that an active cell cycle enhances neonatal engraftment.

We also hypothesized that HSCs from different developmental stages home to different hematopoietic tissues in neonates and adults, which likely has a significant impact on long-term engraftability. We transplanted LT-HSCs from adult bone marrow into neonatal or adult recipients, and after 15 hours recovered and dissociated multiple tissues and surveyed for donor cells by flow cytometry. We chose to examine the recipients 15 hours post-injection to assess homing prior to proliferation of the donor cells [39-41]. At 15 hours post-transplant, we detected comparable numbers of donor cells in both neonates and adults, indicating that a lack of short-term donor cell survival could not account for the failure of adult LT-HSCs to engraft in neonates. In adult recipients 42% of injected cells were detected in the liver, 48% in the spleen, and 8.7% in the marrow of the leg bones. In neonatal recipients, 88% of injected cells were detected in the liver, 4.3% in the spleen, and 6.9% in the marrow of the leg bones. Very few or no donor cells were found in the thymus, peripheral blood, or lungs of either adults or neonates (Figure 2-7). These data demonstrate that definitive adult-like HSCs home to different tissues in neonates relative to adult recipients, which may underscore the difference observed in long-term engraftment. We also assayed the homing of VE-Cadherin<sup>+</sup> CD45<sup>+</sup> cells from E11.5 AGM but were unable to detect any donor cells at 15 hours post-transplant. This suggests few early embryonic HSCs survive 15 hours post-transplants, and those that survive to contribute to reconstitution are too few to detect.

Figure 2-6

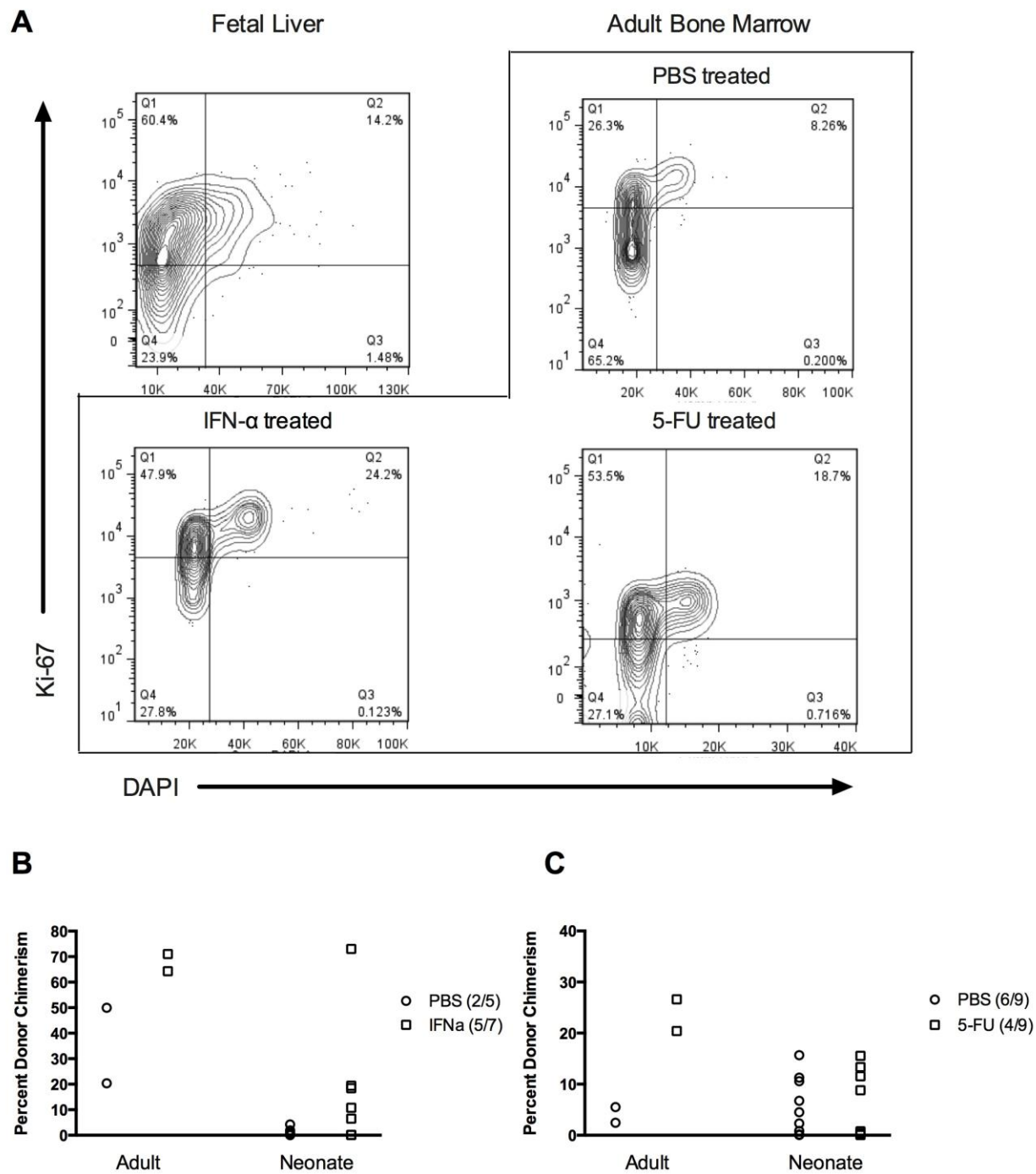


Figure 2-6: Cycling adult LT-HSCs engraft neonates. A) FACS plots for Ki-67 and DAPI expression on E14.5 fetal liver LT-HSCs and sorted bone marrow LT-HSCs from IFN $\alpha$  treated, 5-FU treated, or PBS control

Figure 2-6 (Continued)

adult mice. B) Neonatal and adult recipients were transplanted with 100 bone marrow LT-HSCs from adult mice treated with IFN $\alpha$  (squares) or PBS (circles). Data shown are at 18 weeks post-transplantation. Fisher's exact test p-value = 0.5581. C) Neonatal and adult recipients were transplanted with 100 bone marrow LT-HSCs from adult mice treated with 5-FU (squares) or PBS (circles). Data shown are at 14 weeks post-transplantation.

Figure 2-7

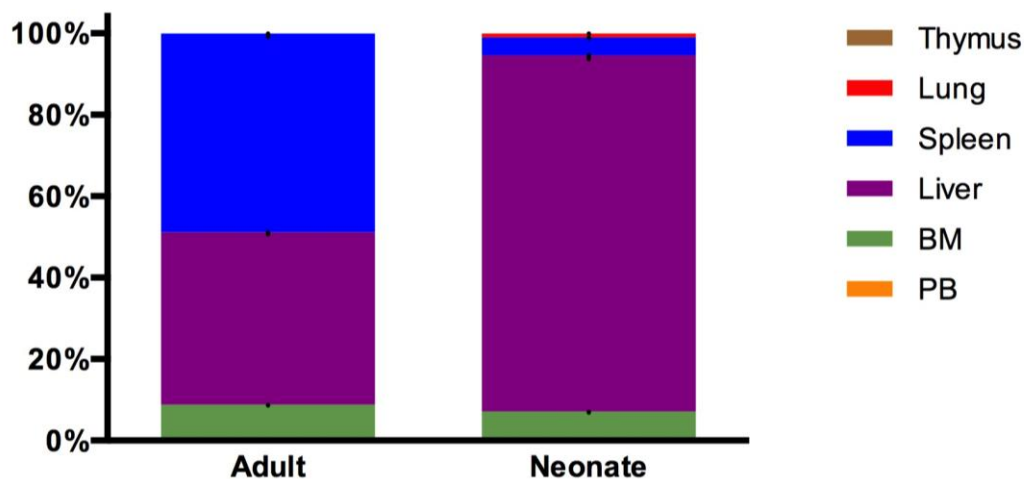


Figure 2-7: Homing of adult LT-HSCs differs in adult and neonatal recipients. Percentages of GFP<sup>+</sup> CD45.2<sup>+</sup> donor adult bone marrow LT-HSCs detected in six tissues in adult and neonatal recipients 15 hours post-transplantation. Data shown is the mean  $\pm$  SEM, representing a total of 5 – 6 mice per condition with data collected in experimental duplicate for adults and triplicate for neonates. Liver and spleen p-value < 0.05; lung, bone marrow (BM), thymus, and peripheral blood (PB) p-value > 0.05 as calculated by the student's t-test.

## 2.4 Discussion

Although prior literature suggests that the neonate harbors a more permissive environment for engraftment of early HSCs [27, 42] and microarray data of early and adult-like HSCs show differentially expressed genes with various functional roles [29], there have been no prior reports directly comparing and quantifying the repopulating cell frequency of HSCs from multiple points during ontogeny in both neonatal and adult recipients. Here we highlight the importance of the recipient in determination of engraftment outcomes, and identify several perplexing differences between the capacity of nascent embryonic and definitive adult-like HSCs to engraft either neonates or adults. Specifically, we show that nascent embryonic HSCs are better suited to engraftment in neonates; conversely, definitive adult-like HSCs, whether harvested from fetal liver or adult bone marrow, more efficiently reconstitute adult hosts. We used the neonatal transplant model to better understand the engraftment requirements of adult HSCs and identified two factors that may modulate neonatal engraftment potential, differential proliferative activity and homing potential. The neonatal transplant assay provides a tool to further our understanding of the factors required for survival, maturation, and function of embryonic HSCs and can be utilized to functionally examine nascent HSC-like cells derived from pluripotent stem cells.

Our data are the first to demonstrate robust long-term multi-lineage hematopoietic reconstitution of unmanipulated, uncultured E10.5 AGM in wild-type murine recipients, thereby establishing that definitive HSCs indeed arise within the embryo at this early stage, but a receptive host is required to observe this functionality. Others have shown that E10.5 AGM and even E9.5 PSp can engraft immunodeficient adult recipients, suggesting that genetic immunodeficiency in adult recipients enables the same permissiveness to engraftment of embryonic cells as we observe in the neonate [19, 22, 23, 43, 44]. Indeed, immaturity of the neonatal immune system may represent a reduced

transplantation barrier for early embryonic HSCs, which express lower levels of MHC Class I molecules, and are thus susceptible to rejection by NK-mediated mechanisms [22]. Interestingly, we have also shown that the neonate is not a more permissive recipient for HSCs from all stages of ontogeny, as purified LT-HSCs from E14.5 fetal liver and adult bone marrow do not engraft neonates as robustly as adults. Adult-like HSCs likely respond to the neonatal environment differently than early HSCs because of the differential expression of genes involved in cytokine-cytokine receptor interaction and membrane composition. The preference of immature HSCs for engraftment in neonates may have clinical parallels in the tendency of umbilical cord blood to more robustly engraft juveniles as compared to adults, a phenomenon which may not depend entirely on cell dose [45-47].

Our study suggests that HSC migration to the bone marrow is coupled to limited neonatal engraftment potential. HSCs harvested from the neonatal liver re-engraft in neonates more robustly than HSCs harvested from the neonatal bone marrow, indicating that either cell intrinsic changes that trigger migration from the liver to the bone marrow also cause decreased neonatal engraftment, or that migration to the bone marrow induces an alteration in HSCs that persists as the animal matures, as adult bone marrow HSCs yield no or low level engraftment in neonates. When we surveyed homing of adult bone marrow HSCs in neonatal and adult recipients, we found most donor cells in the liver in neonates and the liver and spleen in adults.

Although we detected similar numbers of donor cells in neonates and adults, and a comparable percent of donor cells homed to the bone marrow in neonates and adults 15 hours post-injection, long-term engraftment of adult bone marrow HSCs in neonates was very poor. Several potential explanations can be entertained to account for this. Coupled with our finding that fetal liver HSCs engraft neonates more robustly than adult bone marrow HSCs, the evidence suggests that the quiescent state of adult HSCs may not be suitable for engraftment in neonatal recipients. The variable engraftment

data from adult donor HSCs exposed to IFN $\alpha$  and 5-FU suggest the underlying mechanism maybe more complex. Recent work defines two distinct niches for cycling and quiescent HSCs in adult bone marrow, which may be present in different ratios or non-existent in the developing neonatal bone marrow [48]. Additionally, our neonatal engraftment conditions permit only sub-lethal doses of irradiation, whereas engraftment of adults is typically measured after lethal doses of irradiation. The sub-lethal irradiation of neonates may not generate a sufficiently strong homeostatic drive to induce substantial proliferation of the otherwise quiescent adult HSCs, especially considering the presence of competing actively cycling neonatal HSCs.

In conclusion, we have used the neonatal transplant model to functionally assay the engraftment potential of emerging early embryonic HSCs, which go undetected when using wild-type adult recipients. Future studies using the neonatal transplant model may allow us to discover new populations in hematopoietic tissues from other points in ontogeny that also engraft neonatal but not adult recipients. Additionally, neonates may provide a unique environment to functionally assay hematopoietic derivatives of pluripotent stem cells.

## 2.5 Materials and Methods

### Animals

C57BL/6 and B6.SJL mice were purchased from Jackson Laboratories and Taconic and bred in house. UBC-GFP mice were purchased from Jackson Laboratories (strain #004353) [49]. Experiments were carried out with IACUC approval from CHB.

## Bone Marrow Preparations

Femurs and tibias from neonatal and adult mice were isolated then crushed with mortar and pestle in PBS and strained through a 70  $\mu$ m filter. The sample was incubated with red blood cell lysis buffer on ice for 20-30 minutes then resuspended in PBS for transplantation or PBS with 2% serum for flow cytometry.

## Embryonic Tissue Preparations

Embryonic day was determined by counting the day of vaginal plugging as 0.5 days post coitus. Staging of embryos was performed by somite counting. Embryos were collected from timed-mated females at E9.5 (16-26 somites), E10.5 (30-39 somites), E11.5 (40-48 somites), and E14.5 (53-55 somites). The AGM/PSP was dissected, dissociated with collagenase/dispase (1mg/mL in DMEM) for 30-60 minutes at room temperature, and strained through a 70  $\mu$ m filter. The fetal and neonatal liver was dissected and mashed over a 70  $\mu$ m filter. The sample was incubated with red blood cell lysis buffer on ice for 20-30 minutes. Cells were resuspended in PBS for transplantation or PBS with 2% serum for cell sorting.

## Bone Marrow Transplantation

Donor and recipient cells were distinguished by CD45.1 and CD45.2. Adult recipients were either conditioned with a lethal dose of irradiation, 10 Gy total split by 3 hours, and received  $3 \times 10^5$  cells from whole bone marrow as helper or sub-lethally conditioned with a 3.5 Gy or 6.5 Gy dose of irradiation and did not receive helper cells. Donor cells were injected via the tail vein. Neonatal recipients, 1 – 2 days old, were conditioned with a sub-lethal single dose of irradiation, 3.5 Gy, and received no helper cells. Donor cells were injected via the facial vein. Post-transplantation, mice were kept on antibiotic-treated drinking water. Engraftment was monitored in adult recipients starting 4

weeks post-transplant and in neonatal recipients starting 6 weeks post-transplant. Neonatal recipient analysis was delayed due to runt size as a result of irradiation. Continued engraftment was monitored every 4 weeks by flow cytometric analysis of peripheral blood.

### Secondary Transplantations

Adult secondary recipients were lethally conditioned with a split dose of irradiation, 10 Gy total dose split by 3 hours. One million to 5 million whole bone marrow cells from the primary recipient were injected via the tail vein into each of 3 to 4 secondary recipients.

### Peripheral Blood Isolation

Peripheral blood samples were collected by retro-orbital bleeds with heparinized capillary tubes. The red blood cells were allowed to settle out of suspension in PBS with 1% dextran and 0.25M EDTA at room temperature. The buffy layer was pelleted and resuspended in red blood cell lysis buffer on ice for 20-30 minutes to remove lingering red blood cells. The remaining leukocyte enriched samples were prepared for flow cytometry.

### FACS Analysis and Sorting

Multicolor FACS analysis was carried out on a 5-laser-LSRII or LSRFortessa flow cytometer (Beckton-Dickinson), and sorting was performed on a FACS-Aria+UV (Beckton-Dickinson). Leukocyte enriched peripheral blood cells were stained on ice for 15-30 minutes with the following antibody cocktail for lineage analysis: CD45.1 – FITC, CD45.2 – PE/Cy7, Ter119 – PE/Cy5, Gr1 – PE, Mac1 – Alexa700, B220 – Pacific Blue, CD19 – APC/Cy7, CD3 – APC, and propidium iodide (Figure 2-8) and for homing: CD45.1 – PE and CD45.2 – PE/Cy7. The cells were washed and resuspended in PBS with 2% serum.



Figure 2-8

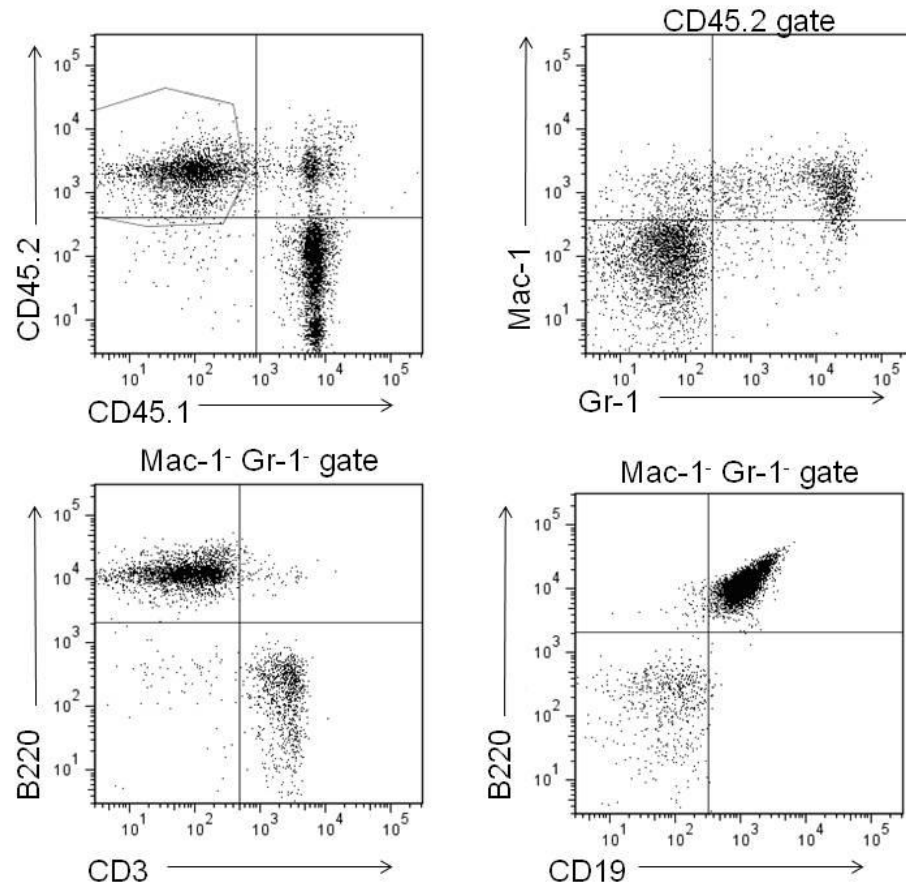


Figure 2-8: Analysis of peripheral blood chimerism. After gating on the live leukocytes, percent donor chimerism was determined by analysis of CD45.2 versus CD45.1. The donor cells were then analyzed for lineage contribution, first Mac-1 versus Gr-1 followed by B220 versus CD3 and B220 versus CD19 within the Mac-1<sup>-</sup> Gr-1<sup>-</sup> quadrant.

To assay the proliferative state of HSCs, isolated HSCs were fixed and permeabilized using the BD Cytofix/Cytoperm kit according to the manufacturer's protocol. DAPI was used as a nuclear stain and Ki-67 – FITC marked proliferating cells. The cells were washed and resuspended in PBS with 2% serum.

To isolate HSCs from bone marrow, samples were stained with biotinylated antibodies against Mac1, Gr-1, B220, CD3, and Ter-119, then lineage depleted with anti-biotin MACS beads. The remaining cells were stained with the following antibody cocktail on ice for 15-30 minutes: Lineage (Mac1, Gr1, B220, CD3, Ter119) – Alexa450, Sca1 – PerCP-Pc5.5, c-Kit – APC/Cy7, CD150 – PE/Cy7, CD48 – PE, and DAPI (Figure 2-9). Preparations for E14.5 fetal liver were identical to bone marrow except for the use of Mac1 in the lineage cocktail. To isolate prospective HSCs from E11.5 AGM, cells were stained with a purified VE-Cadherin antibody followed by a goat anti-rat Alexa680 secondary antibody then CD45 – PE and propidium iodide (Figure 2-4 A).

Figure 2-9

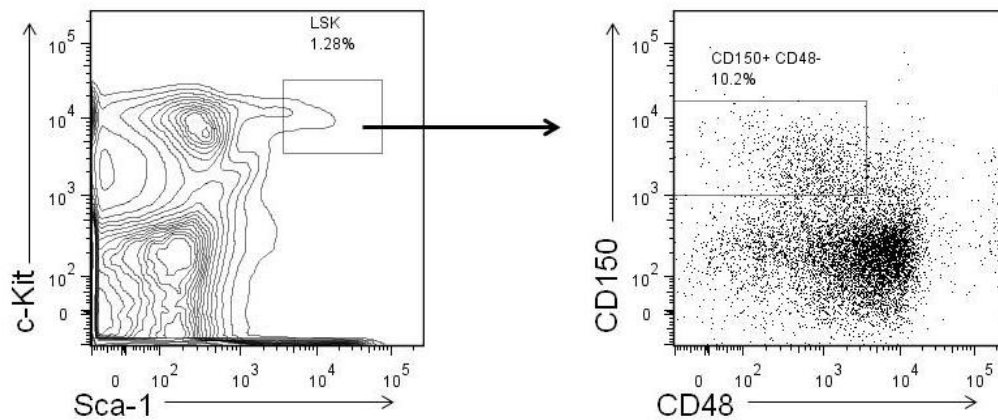


Figure 2-9: Isolation of bone marrow LT-HSCs. After gating on the live lineage negative cells, LSK cells were gated on in the c-Kit versus Sca-1 plot. Within the LSK gate, CD150<sup>+</sup> CD48<sup>-</sup> cells were gated on for sorting.

## Limiting Dilution Analysis (LDA)

LDA was performed online using software provided by WEHI bioinformatics <http://bioinf.wehi.edu.au/software/elda/index.html> [50]. In all cases, the threshold for engraftment was 1% donor chimerism at 18 weeks post-transplantation.

## HSC Proliferation

To induce HSC proliferation, adult mice were treated with one dose of IFN $\alpha$  (10,000 units given subcutaneously) 24 hours prior to HSC isolation. Alternatively, adult mice received one dose of 5-FU (150 mg kg<sup>-1</sup>) by intraperitoneal injection 5 days prior to HSC isolation.

## Homing

VE-Cadherin<sup>+</sup> CD45<sup>+</sup> cells were isolated from E11.5 AGM of UBC-GFP embryos. LT-HSCs (Lineage<sup>-</sup> Sca-1<sup>+</sup> c-Kit<sup>+</sup> CD150<sup>+</sup> CD48<sup>-</sup>) were isolated from adult bone marrow of UBC-GFP mice. Approximately 10<sup>4</sup> isolated cells were transplanted into conditioned B6.SJL neonatal and adult recipients. Transplanting purified LT-HSCs in addition to injecting a small number of cells reduces the likelihood of donor cells being trapped in the lung. Adult recipients received 3 x 10<sup>5</sup> helper cells from B6.SJL bone marrow. At 15 hours post-injection, the lung, peripheral blood, bone marrow from the long bones of the leg, spleen, liver, and thymus were collected. Peripheral blood and bone marrow from adult recipients were subjected to red blood cell lysis for 20-30 minutes on ice. Solid tissues were mashed over a 70  $\mu$ m filter. Cells from the collected tissues were stained with antibodies for CD45.1 and CD45.2. Donor cells were detected using two markers: CD45.2 and GFP expression.

## Statistical Analysis

Fisher exact test was used for statistical analysis of the difference in frequency of engraftment. In cases where a majority of the animals were engrafted, the student's t-test was used for statistical

analysis of the engraftment levels. The LDA algorithm performed statistical analysis on the limiting dilution transplants.

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## **Chapter 3**

### **Primitive Fate of RBCs Derived from Human Pluripotent Stem Cells**



### 3.1 Abstract

Human pluripotent stem cell (hPSC) derivatives have the potential to provide new sources of cells for treatment of hemoglobinopathies and sickle cell disease as well as provide platforms for drug screening and *in vitro* modeling, though the hurdle of generating definitive adult-like red blood cells (RBCs) must first be overcome. Current directed differentiation methods produced only primitive RBCs. We compared the directed differentiation of 11 different hPSC lines into RBCs using three different published protocols and optimized the most robust and reproducible cell line/protocol combination. We then altered the differentiation protocol to include over-expression of key erythroid transcription factors, BCL11A and KLF1, and co-culture on stromal cells, but we observed no change in cell fate and produced only primitive RBCs. We also transplanted early progenitors into a supportive *in vivo* environment, but we were unable to detect donor erythroid cells. The system we have optimized is a good platform for larger scale screens of other transcription factors, stromal lines, or chemicals that may promote and support the definitive cell fate.

### 3.2 Introduction

Since the first demonstration of mutant hemoglobin over a half century ago, hundreds of mutations in the  $\alpha$ - and  $\beta$ -globin loci have been identified. Many mutations are silent or do not present a clinical phenotype, but a few cause severe phenotypes. One of the most common hemoglobinopathies caused by these mutations is sickle cell anemia (SCA). SCA is caused by a point mutation in the sixth codon of the  $\beta$ -globin gene, which predisposes hemoglobin to polymerization,

inducing rigidity and structural deformations of RBCs recognized by a characteristic sickling [1, 2].

Sickled RBCs cause vaso-occlusion, which leads to painful crises, organ damage, and premature death [3-8].

Current treatments for SCA are limited. Hydroxyurea reduces the frequency of painful crises by lowering white blood cell counts, thereby reducing cellular sludging, and increasing production of  $\gamma$ -globin which reduces RBC sickling [9, 10]. RBC transfusions temporarily increase the number of circulating normal RBCs, but frequent RBC transfusions result in sickle antibody sensitization and iron accumulation, which itself causes organ damage [11-17]. Bone marrow transplants are a curative option but are riskier than RBC transfusions, given the potential for graft failure, immunologic complications like graft-versus-host disease, and considerable risk of morbidity and mortality.

The advent of induced pluripotent stem cell (iPSC) technology gives hope for patient specific RBCs and hematopoietic stem cells that would bypass immunologic complications. Although iPSCs are remarkably similar to embryonic stem cells (ESCs) in pluripotent function, many studies have identified subtle but potentially significant molecular differences and differences in the potential to differentiate into blood [18-21]. Examination of the blood differentiation potential of 22 hPSC lines generated at multiple institutions showed higher hematopoietic colony forming unit activity and better erythroid differentiation efficiency from hESCs than hiPSCs [22]. Here, we focused on hPSCs directly differentiated into RBCs to improve our understanding of erythropoiesis, provide new therapeutic options for hemoglobinopathies, and create a platform to screen anti-sickling compounds.

The largest hurdle in harnessing the power of PSCs derived RBCs is generating definitive RBCs instead of the readily generated primitive RBCs [23-31]. Generating definitive RBCs is necessary for disease modeling and drug screening because hemoglobinopathies and SCA do not manifest in primitive

RBCs [32, 33]. For cell therapies, it is unknown how patients would respond to treatment with primitive cells and how effective primitive cells would be in alleviating symptoms of the disease [34, 35].

Definitive RBCs have many unique features that distinguish them from primitive RBCs, but only a few are well studied and easy to assay. Primitive RBCs may not enucleate, are larger in diameter, and express  $\epsilon$ -globin [36]. Definitive RBCs are enucleated and approximately 6-8  $\mu\text{m}$  in diameter [37]. The earliest definitive RBCs express  $\gamma$ -globin while  $\beta$ -globin is expressed in the adult definitive RBCs [36]. While the expression of different hemoglobin genes is not causative of a change in cell fate, we use it here as a marker of the primitive and definitive states. It has not yet been shown if the type of hemoglobin expressed can be uncoupled from the cell fate.

Several  $\gamma$ -globin repressors have been identified in mouse studies, GATA-1, OCT-1, and BCL11A, that may promote globin switching by reducing competition between  $\gamma$ -globin and  $\beta$ -globin for the locus control region [38-45]. Genome wide association studies found BCL11A and KLF1 to be associated with hereditary persistence of fetal hemoglobin [38-40, 46]. Further work suggested a direct role of BCL11A, specifically the L and XL isoforms, in repressing  $\gamma$ -globin [47, 48]. BCL11A is activated by KLF1, which itself activates  $\beta$ -globin [46, 49, 50].

We tested the hypothesis that hPSC derived RBCs could transition from the primitive to definitive fate, and we found the cell fate of hPSC derived RBCs to be unaltered by a variety of approaches. There are two possible explanations: 1) the precursor of primitive RBCs cannot be converted into a definitive RBC precursor under tested conditions or 2) the precursors that give rise to definitive RBCs cannot emerge under standard tissue culture conditions.

### 3.3 Results

We tested three published protocols for directed differentiation of hPSCs into RBCs with 10 hiPSC lines and 1 hESC line originating from a variety of somatic cell types including fibroblasts, cord blood, and mesenchymal stem cells (Figure 3-1) [25, 29, 30]. We examined the general hematopoietic differentiation potential of each line by the ability to form CFUs, the diameter of the hPSC derived RBCs, the capacity for expansion during differentiation, and the amount of hemoglobin mRNA present (Table 3-1). Regardless of the hPSC line or protocol used, the resulting RBCs were primitive. With some modifications, the main protocol from Lu *et al.* generated large numbers of RBCs through a hemangioblast expansion phase followed by erythroid differentiation and expansion. We found recombinant HoxB4 was unnecessary for both directed differentiation and generation of large numbers of RBCs. At the end of the differentiation, many of the hPSC lines made RBCs but not to the same degree in terms of the maturity and number of RBCs produced (Figure 3-2). MSC-iPS1, a mesenchymal stem cell derived hiPSC line, was the most robust of all tested hPSC lines and therefore was used for all other studies.

Figure 3-1

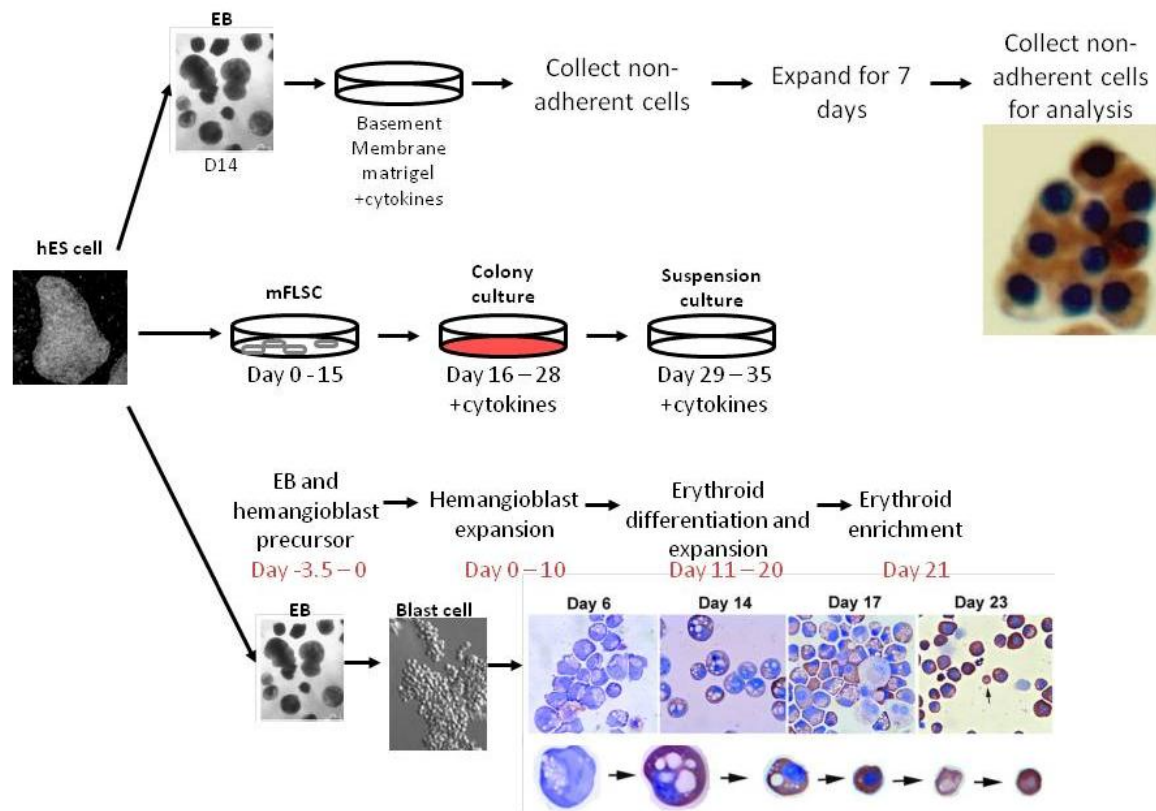


Figure 3-1: hPSC differentiation protocols. Schematic representation of the Chang protocol (top), Ma protocol (middle), and Lanza protocol (bottom). Modified from Lu *et al.*, Chang *et al.*, and Ma *et al.*

Table 3-1: Comparison of hematopoietic differentiation of multiple hPSC lines. The CFU column displays the types of colonies formed from each hPSC line. The cell diameter (size) was determined from derivatives of the Chang protocol. Expansion was calculated as output cells/input cells. Hemoglobin expression was determined by qPCR for  $\epsilon$ -,  $\gamma$ -, and  $\beta$ -globin.

Cell Line	CFU	Size ( $\mu\text{m}$ ) after Chang	Expansion in Chang	Expansion in Ma	Expansion in Lanza	Hemoglobin Expression
H1	E, M, GM	21-25	N/D	7-fold	2.97-fold	Inconsistent
hFib2-iPS5	Almost none, M, G	N/D	N/D	N/D	0.38-fold	Almost none
MRC5-iPS20	M, G, GM, GEMM	22	N/D	N/D	0.03-fold	Moderate
MSC-iPS1	M,G, GM	28	2.9-fold	N/D	0.65-fold	Weak
MRC5-iPS7	M, G, GM	20	2.75-fold	N/D	0.1-fold	Moderate
BJ1-iPS2	N/D	N/D	2.5-fold	N/D	N/D	Moderate

Figure 3-2

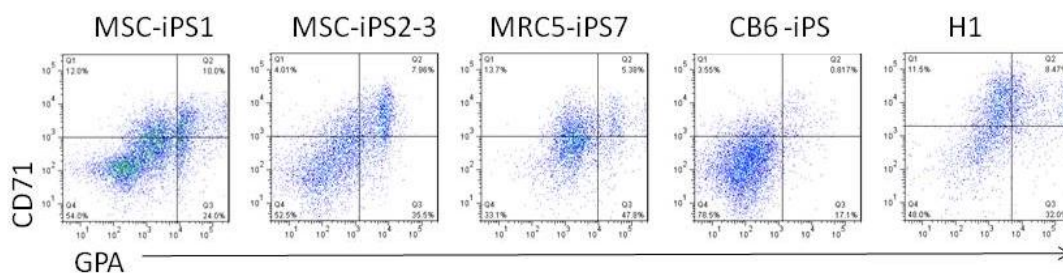


Figure 3-2: Expression of erythroid markers. FACS analysis of erythroid markers CD71 and GPA on cells from the Lanza protocol originating from 5 hPSC lines.

We further characterized the hPSC derived RBCs. RBCs arose in colonies sometimes during the hemangioblast expansion phase but more often during the erythroid specification phase (Figure 3-3 A). At the end of the protocol, the cultures consisted mostly of nucleated orthochromatic erythroblasts and macrophages (Figure 3-3 B). Likely due to the retention of the nucleus, the average diameter of the hPSC derived RBCs was 17.6  $\mu\text{m}$ , which is larger than *in vivo* RBCs (Figure 3-3 C). FACS analysis of RBC surface antigens CD71 and GPA showed maturation of hPSC derived RBCs (Figure 3-3 D). As expected from the data in Lu *et al.*, the RBCs derived from multiple hPSC lines expressed embryonic ( $\epsilon$ ) and fetal ( $\gamma$ ) hemoglobins but not the adult  $\beta$ -globin when both bulk cultures and single cells were analyzed (Figure 3-3 E and F). Cord blood controls expressed both  $\gamma$ -globin and  $\beta$ -globin, but no  $\epsilon$ -globin (Figure 3-3 E and G). Closer analysis of hemoglobin expression at the single cell level showed a gradient of  $\epsilon/\gamma$  expression (Figure 3-3 F). Examination of hemoglobin expression during the hemangioblast expansion phase showed that the ratio of expressed hemoglobins is established early during differentiation (Figure 3-4). In addition to hemoglobin expression, we also used Ep1 to assess the developmental stage of hPSC derived RBCs [51]. Stamatoyannopoulos's group reported Ep1 expression was more prevalent on fetal RBCs than adult RBCs. Less than 1% of cord blood cells expressed Ep1, whereas 3.5% of hPSC derived RBCs expressed Ep1, suggesting cord blood and hPSC derived RBCs could be of similar developmental stages even though they express different hemoglobins (Figure 3-5). We confirmed the Ep1<sup>+</sup> cells were erythroblasts by CD71 and GPA expression.

In light of many papers demonstrating plasticity of cell fate and the power of key transcription factors to alter cell fate [52-55], we analyzed the expression of a panel of erythroid transcription factors and tested the potency of two key factors, BCL11A and KLF1. The ratio of expressed hemoglobins was used as an indicator of primitive versus definitive cell fate. During the early stages of directed differentiation, we found no clear patterns in the mRNA levels of erythroid transcription factors, suggesting a loosely regulated or stochastic process (Figure 3-6).

Figure 3-3

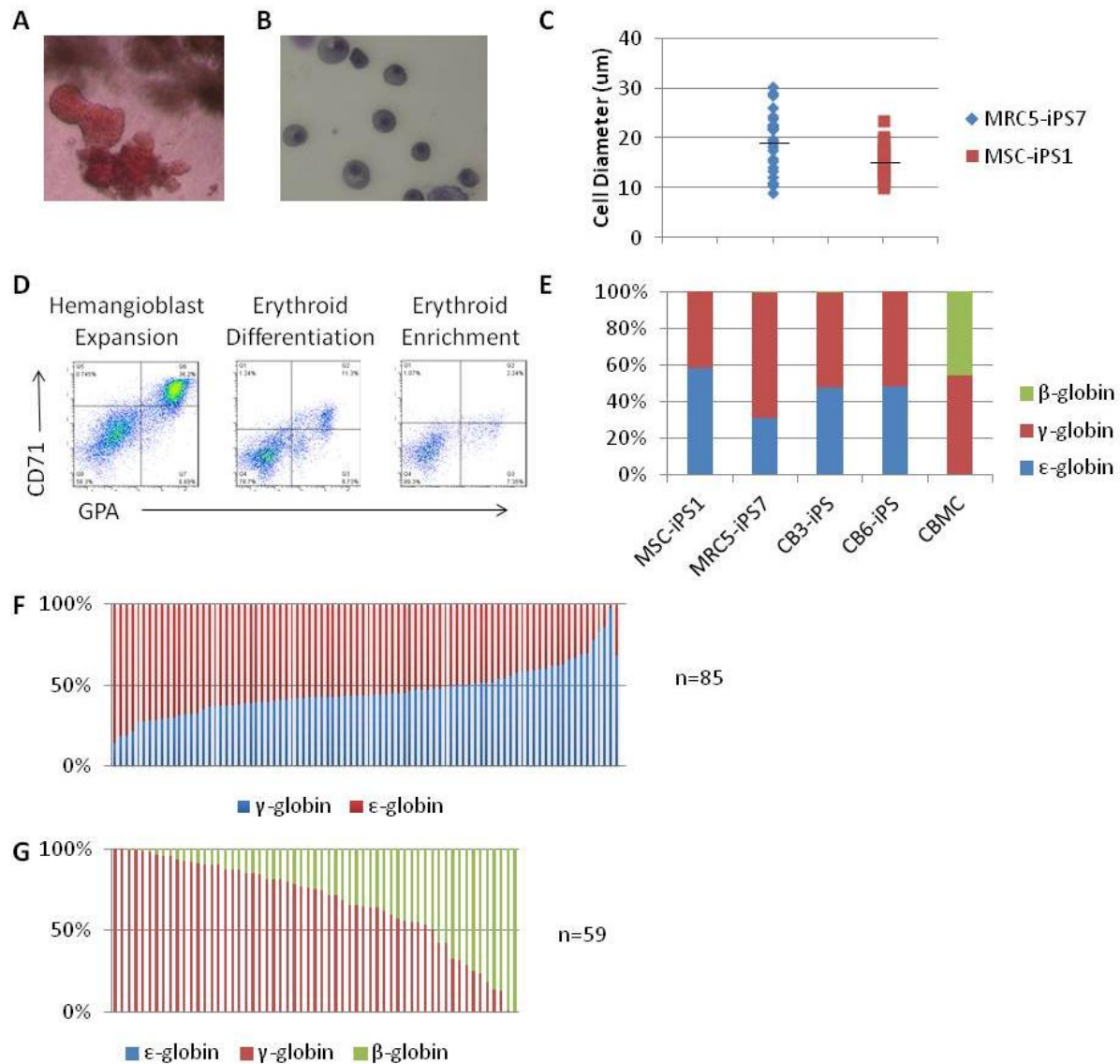


Figure 3-3: Primitive hPSC derived RBCs from Lanza protocol. A) Bright-field image of RBC clusters during the erythroid differentiation phase. B) Wright-Giemsa staining on cytopins from the erythroid differentiation phase show orthochromatic erythroblasts. C) Diameter of cells derived from two hiPSC lines at the end of the erythroid differentiation phase. D) FACS analysis of differentiating cells. E) Hemoglobin expression in derivatives of 4 hiPSC lines at the end of the differentiation protocol. Expression was determined by qPCR and compared to cord blood mononuclear cells (CBMC). F) Single-



Figure 3-3 (Continued)

cell qPCR analyzing hemoglobin expression in MSC-iPS1 derived RBCs at the end of the differentiation protocol. G) Single cell qPCR analyzing hemoglobin in CD71 enriched cord blood.

Figure 3-4

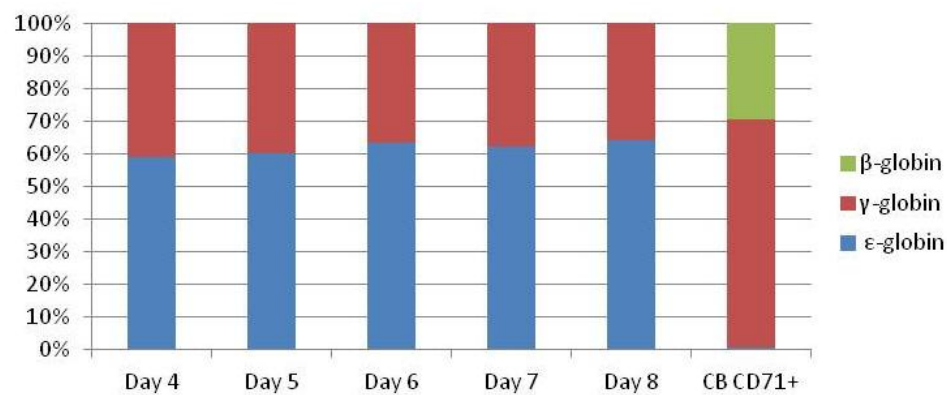


Figure 3-4: Hemoglobin expression during hemangioblast expansion. A time course analysis of the hemoglobins expressed, as determined by qPCR, during the hemangioblast expansion phase with MSC-iPS1 cells. Expression is compared to CD71 enriched cord blood mononuclear cells (CB CD71<sup>+</sup>).

Figure 3-5

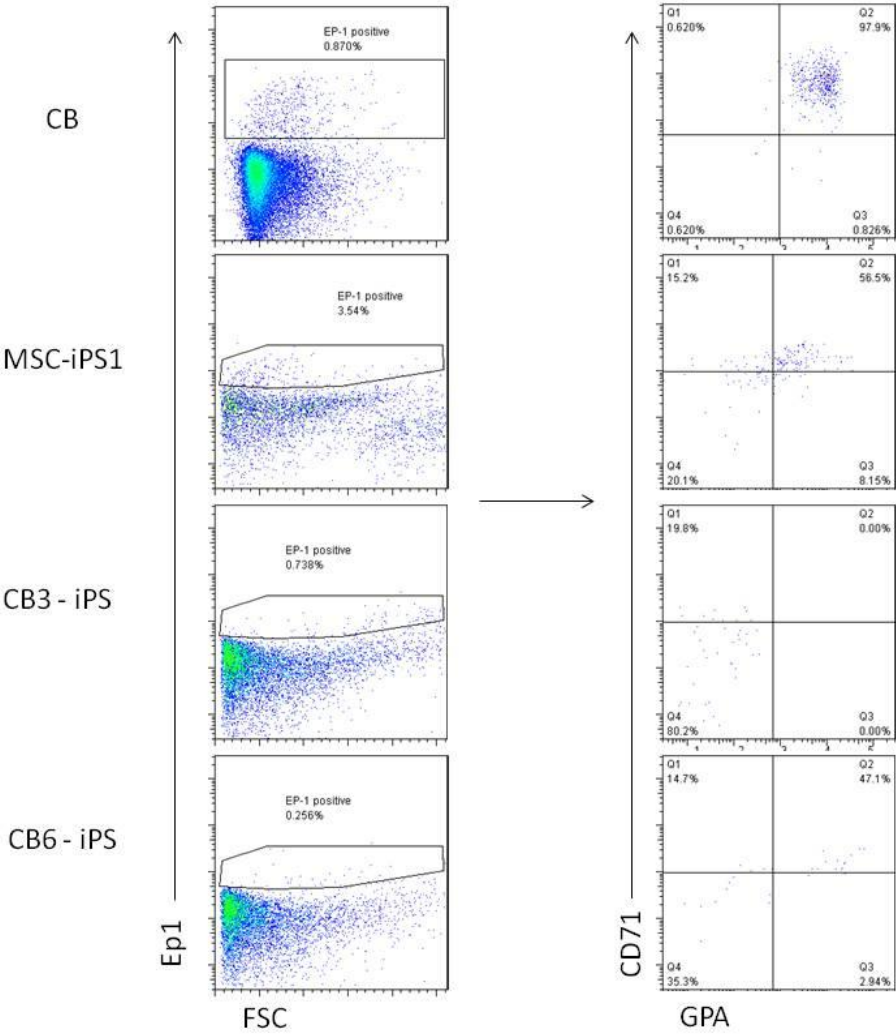


Figure 3-5: Expression of developmental marker Ep1. FACS analysis of Ep1 on cord blood cells compared to RBCs derived from three hPSC lines and subsequent analysis of CD71 and GPA within the Ep1<sup>+</sup> gate. CB – cord blood.

Figure 3-6

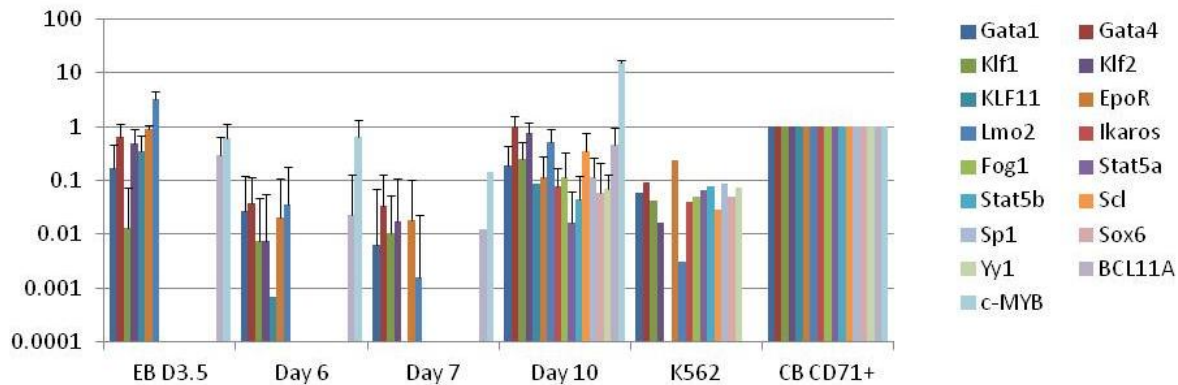


Figure 3-6: Erythroid transcription factors during differentiation. mRNA levels of erythroid transcription factors at the end of the embryoid body phase (EB D3.5) and at time points during the hemangioblast expansion phase determined by qPCR.

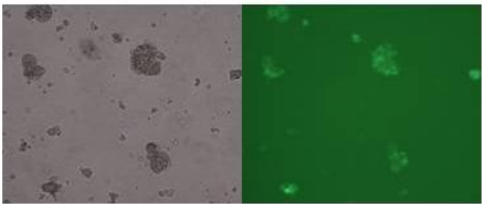
We developed a method to introduce exogenous transcription factors at the beginning of the hemangioblast expansion phase (Figure 3-7 A). We hypothesized that exogenous transcription factors would need to be expressed early during differentiation to alter the hemoglobin expression pattern that seemed to be established in the hemangioblasts. GFP<sup>+</sup> cells expressing CD71 and GPA demonstrate that the exogenous factors were expressed in the hPSC derived RBCs (Figure 3-7 B). Increasing expression of two known key erythroid transcription factors BCL11A and KLF1 by 1.7- to 7.5-fold highlighted interesting relationships between the two factors (Figure 3-7 C). We examined the effect of over-expressing BCL11A-L and BCL11A-XL as both isoforms are expressed in definitive RBCs [47, 48]. KLF1 levels dropped 6.3-fold when BCL11A-L was over-expressed and dropped 12.5-fold when BCL11A-XL was over-expressed, suggesting a negative feedback loop as KLF1 is known to activate BCL11A. BCL11A-XL seems to be a more potent repressor of KLF1 than BCL11A-L. The ratio of expressed hemoglobins in the bulk culture was largely unaltered by the over-expression of KLF1, BCL11A-L, or BCL11A-XL (Figure 3-7

D). Single cell analysis showed no change in hemoglobin expression after KLF1 over-expression (Figure 3-7 F). However, over-expression of KLF1 along with BCL11A-L produced 1 cell out of the 44 examined with 94%  $\beta$ -globin (Figure 3-7 G); as this was not seen in controls, it indicates that KLF1 and BCL11A-L may activate  $\beta$ -globin expression and repress  $\gamma$ -globin expression during differentiation of hPSCs, but it is a rare event.

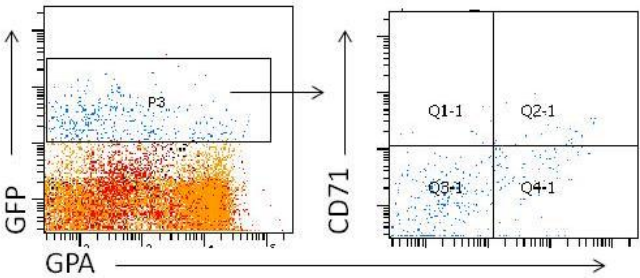
One possible explanation for the observed lack of change in hemoglobin expression is that the proper microenvironment providing cell-cell interactions and additional growth factors are missing [56-58]. To test this hypothesis, we cultured differentiating cells on human fetal liver and bone marrow stromal lines. There was no detectable difference in hemoglobin expression after 13-15 days of co-culture, suggesting that the cells were not responsive to the added microenvironment or that the stromal lines did not provide the cell-cell interactions and growth factors necessary to convert the cells to the definitive fate (Figure 3-8).

Figure 3-7

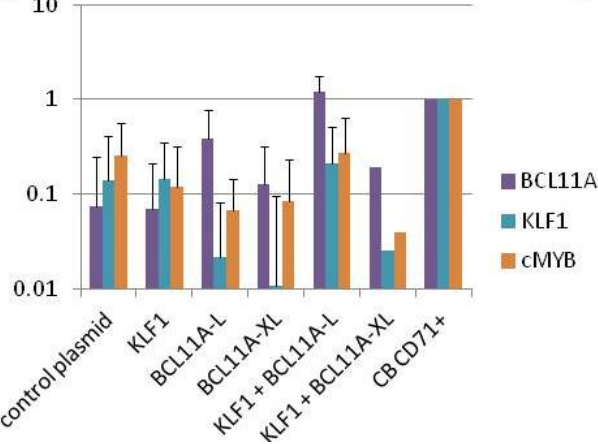
A



B



C



D

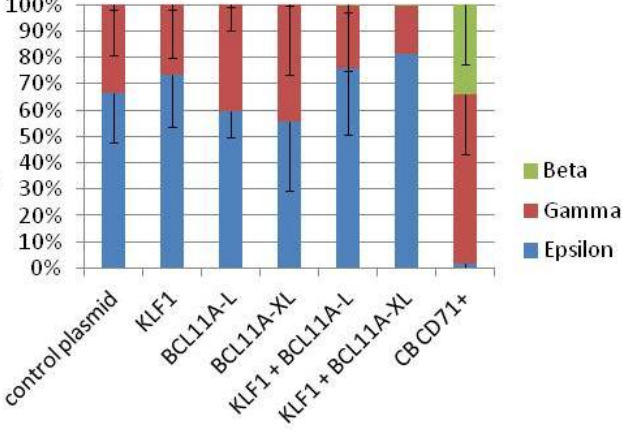


Figure 3-7 (Continued)

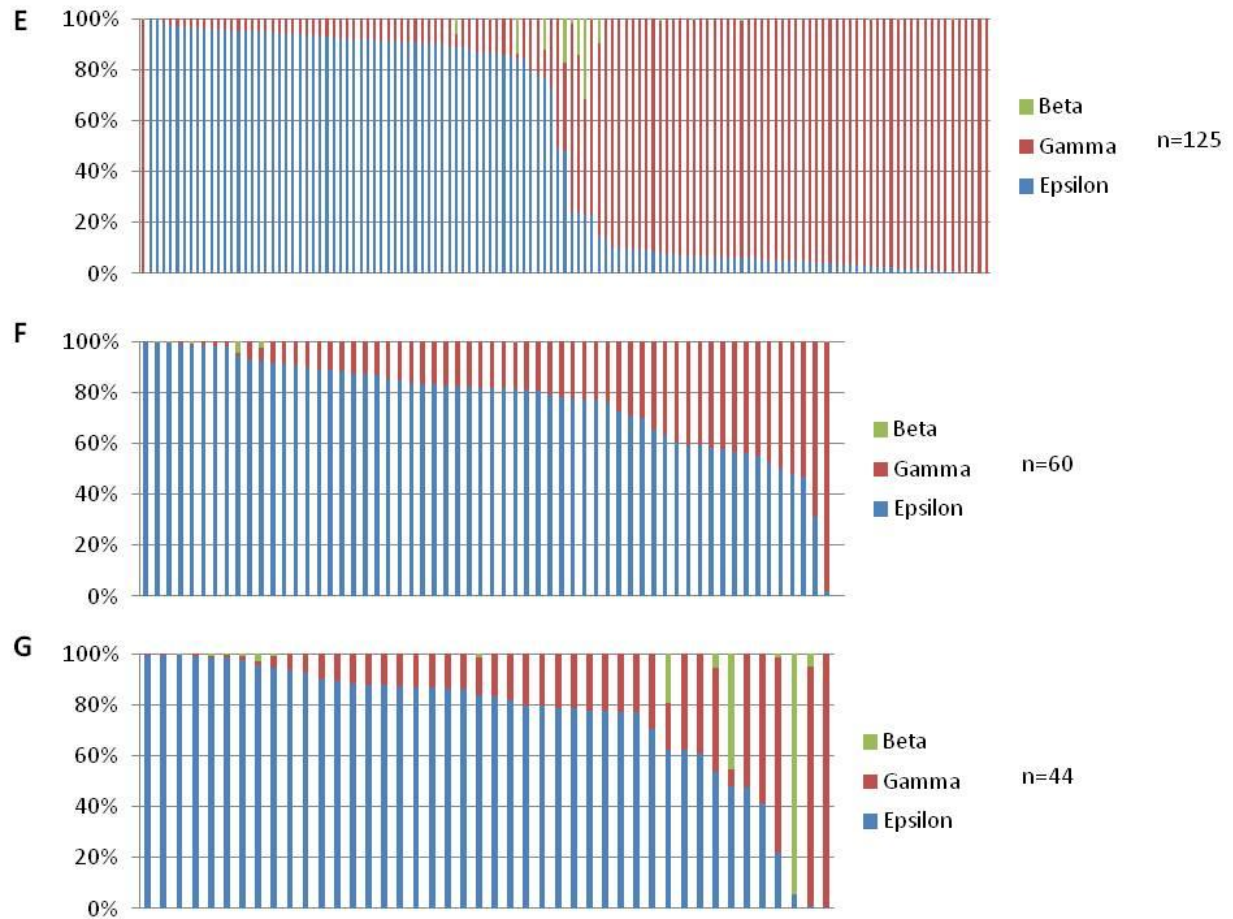


Figure 3-7: Over-expression of key erythroid transcription factors. A) Bright-field and fluorescent image of differentiating MSC-iPS1 infected with a GFP virus. B) FACS analysis of GFP<sup>+</sup> cells also expressing CD71 and GPA. C) Expression of key erythroid transcription factors after over-expression with exogenous KLF1 and/or BCL11A determined by qPCR. D) Expression of hemoglobins after over-expression of KLF1 and/or BCL11A determined by qPCR. CB – cord blood. Single cell qPCR analysis of expression of hemoglobin genes in differentiating cells infected with E) control virus, E) KLF1 virus, or G) KLF1 and BCL11A-L virus.

Figure 3-8

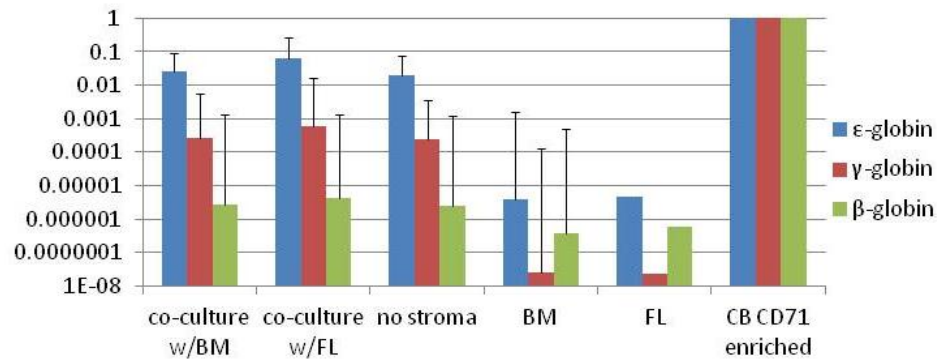


Figure 3-8: Co-culture during differentiation. Hemoglobin expression of differentiating MSC-iPS1 cells co-cultured on bone marrow (BM) or fetal liver (FL) stromal cells. No stroma – differentiating cells without any co-culture. qPCR was used to determine expression of each hemoglobin, which was normalized to expression in cord blood cells enriched for CD71 expression.

We next determined if hematopoietic stem/progenitor cells derived from hiPSCs by the modified Lu *et al.* protocol have the potential to generate definitive RBCs in the hypothesized most permissive environment, immunodeficient neonates engineered to express human SCF, GM-CSF, and IL-3. First, we assessed the potential for and function of hematopoietic stem/progenitor cells. FACS analysis showed the number of CD34<sup>+</sup> CD38<sup>-</sup> cells peaked at day 7 of the hemangioblast expansion phase (Figure 3-9 A). CFU analysis on bulk differentiation cultures showed more colonies forming on or before day 6 of the hemangioblast expansion phase with a small number of GEMM colonies present at all days analyzed (Figure 3-9 B). These data suggest the presence of hematopoietic stem/progenitor cells between days 4 – 8 of the hemangioblast expansion phase. We transplanted 1-2 x 10<sup>6</sup> cells from day 6 and day 8 of the hemangioblast expansion phase into each of 36 NSG or NSG-SGM3 neonatal recipients via intrahepatic or intravenous injection and compared engraftment to 50,000 CD34<sup>+</sup> enriched cord

blood cells. We used two different antibody clones for CD45 to confidently identify low level human chimerism. At 6 months post-transplant, the cord blood cells had engrafted the mice, but we detected no consistent or robust donor chimerism as early as 2 weeks or as late as 6 weeks weeks/months post-transplant in mice that received hPSC derivatives (Figure3-10). There are many possible explanations for the lack of donor chimerism. Coupled with the CFU analysis, we suspect short-term survival of the donor cells is low, thus we cannot detect the presence of donor cells or their progeny even at 2 weeks post-transplant.



Figure 3-9

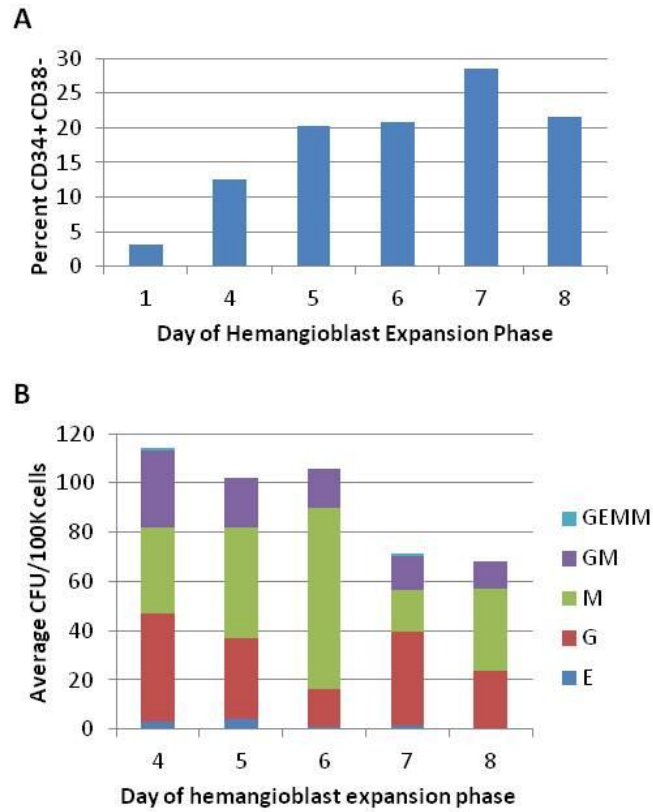


Figure 3-9: Emergence of hematopoietic stem/progenitor cells. A) Percent of hemangioblast expansion culture that is CD34<sup>+</sup> CD38<sup>-</sup> as determined by FACS analysis at different time points. B) Hematopoietic colony forming potential of cells during the hemangioblast expansion phase.

Figure 3-10

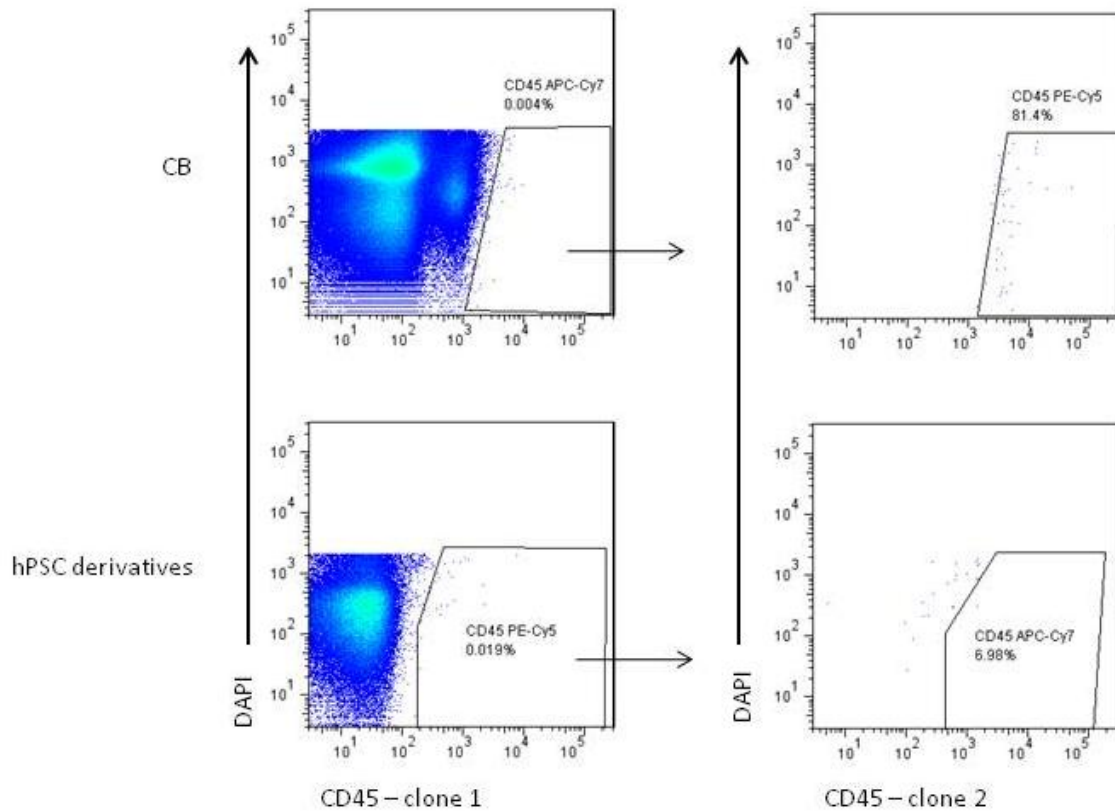


Figure 3-10: Neonatal xenotransplantation. Cord blood CD34<sup>+</sup> cells and hPSC derivatives from the hemangioblast expansion phase were transplanted into neonatal NSG mice. FACS analysis shows the percent donor chimerism in the bone marrow after 2 weeks. CB – cord blood

### 3.4 Discussion

Our data is suggestive of a hypothesis where embryonic and definitive RBCs stem from two distinct stem/progenitor populations that have limited plasticity [36, 37]. In comparing three previously published protocols for the directed differentiation of hPSCs towards RBCs, we confirmed the hPSC

derived RBCs to be primitive in all protocols, regardless of the hPSC line the RBCs were derived from. Using the Lanza protocol, which was the only one that generated large numbers of RBCs, we were unable to alter RBC fate, measured by the ratio of expressed hemoglobins, by over-expressing key erythroid transcription factors, co-culturing on stromal lines, and transplanting derived cells into a hypothesized supportive *in vivo* environment.

As expected from the literature, RBCs generated through the Lanza protocol were primitive, expressing similar amounts of  $\epsilon$ -globin and  $\gamma$ -globin but negligible amounts of  $\beta$ -globin. By FACS, we observed the cells maturing through erythropoiesis, from  $CD71^+ GPA^+$  to  $CD71^- GPA^+$ . At the end of the differentiation protocol, the cells were still nucleated, which is related to the observed large cell diameter. This phenotype was consistent regardless of the original hPSC line used. iPSCs have a bias towards differentiating into the lineage of the starting somatic tissue by retaining cellular memory after reprogramming [19]. We directly differentiated two cord blood derived hiPSC lines into RBCs, but the hemoglobin expression pattern was nearly identical to that of all other tested hiPSC lines, suggesting the cells retain no memory of their definitive state before reprogramming. Similar resetting of ageing genes has been reported in the context of iPSCs generated with samples from Parkinson's patients [59].

Our strategy to alter the fate of the hPSC derived RBCs was to provide new signals early during differentiation while the cells were still in the reported hemangioblast phase. We hypothesized that the cells would be less responsive to fate change cues once they committed to the erythroid lineage. Over-expressing key erythroid transcription factors, BCL11A and KLF1, that are known to be involved in hemoglobin regulation did not consistently or robustly affect the ratio of expressed hemoglobins [46, 47, 49]. It is possible that additional factors such as Sox6 and c-Myb are required to alter the fate of embryonic RBCs [38, 60-63]. The system we developed for viral infection during directed RBC differentiation can be used to screen other key transcription factors, co-factors, and epigenetic

regulators that will come out of future analysis of published microarrays [64-66]. However, determining which transcription factors to screen may require preliminary studies as our data shows inconsistent expression of key erythroid transcription factors in the early phases of directed differentiation.

Stromal lines have been shown to enhance the derivation and maintenance of many cell types including hPSCs and hematopoietic stem cells [67-70]. We observed no change in the ratio of expressed hemoglobins when we cultured differentiating cells on hematopoietic supportive human fetal liver or bone marrow stromal lines previously reported to support hematopoietic cells [68, 71]. With recently available microarray data [64, 66], cell-cell interactions and cytokine receptors that differ between embryonic and definitive RBCs and their progenitors could identify stromal lines with supportive of the emergence of definitive RBCs.

In an effort to provide the most supportive environment for the emergence of definitive RBCs, we transplanted a bulk mixed culture of hPSC derived hemangioblasts and RBC progenitors intrahepatically into neonatal immunodeficient mice expressing human SCF, GM-SCF, and IL-3, but we did not detect consistent or robust engraftment. By introducing the cells intrahepatically, we removed the possibility that the cells were unable to home to a hematopoietic tissue. There are many other factors that may have contributed to our observations. Human erythroid engraftment is known to be short-lived, and thus donor erythroid cells may be depleted by 2 weeks post-transplant. Also, the embryonic cells may not survive in the host animal [72, 73].

Collectively, our data supports the hypothesis that the embryonic and definitive erythroid lineages arise from two different stem/progenitor populations. Further work is needed to understand the plasticity of the two populations. The two stem/progenitor populations could be extremely plastic. Culture conditions supportive of the embryonic state may hinder the emergence of extremely plastic definitive stem/progenitor cells, as the definitive cell would rapidly convert to the embryonic fate.

Alternatively, the two stem/progenitor populations may have minimal plasticity, thereby restricting direct conversion or maturation in an environment that supports the emergence of embryonic cells. Stem cell biologists have yet to find the key to mature cells to the definitive fate, as it is easier to return a cell to a state it passed through during development than it is to restrict a differentiating cell to a specific fate that it has no cellular memory of. Adult cells are able to return to a more embryonic state as exemplified by iPSC reprogramming and increased fetal hemoglobin in RBCs after BCL11A knockdown [47, 55, 74]. Although, it is unknown if the fate of the cells, or only the hemoglobin expression, was altered.

Once the technology exists to create definitive RBCs, it can be coupled with SCA-iPSC lines to produce an *in vitro* model of SCA. Three groups have reported the generation of humanized iPSC models of SCA [75-77]. Only one report demonstrated sickling of hiPSC derived RBCs [77]. Other groups used zinc finger nucleases and CRISPR to create genetically corrected SCA-iPSC lines, which can be utilized as controls in screens for anti-sickling compounds [76, 78]. The technology would also enable the production of genetically corrected patient specific hiPSC derived RBCs for patients with no histocompatible donor.

### 3.5 Materials and Methods

#### hPSC Maintenance

hPSCs were maintained on mouse embryonic fibroblasts (MEFs) in hES medium (DMEM-F12 supplemented with 20% knock-out serum replacement, 100  $\mu$ M nonessential amino acids, 50 U/mL penicillin, 50 mg/mL streptomycin, 1 mM L-glutamine, 100  $\mu$ M 2-mercaptoethanol, and 10 ng/mL bFGF).

Before plating MEFs, tissue culture plates were pre-coated with 2% gelatin for 20 minutes at room temperature. MEFs (Global Stem CF-1 irradiated) were quickly thawed at 37°C, centrifuged at 1,200 rpm for 4 minutes, resuspended, and plated in MEF medium (DMEM supplemented with 10% fetal calf serum, 50 U/mL penicillin, 50 mg/mL streptomycin, and 1 mM L-glutamine) at a density of 67,000 MEFs per well of a 6-well plate. MEF plates rested for 12 – 48 hours before hPSCs were plated on top.

hPSCs were passaged every 5 days to maintain cultures with less differentiation. Differentiation was first aspirated away, and then media was replaced with collagenase IV (1 mg/mL in DMEM-F12). After 5 minutes, the enzymatic solution was replaced with DMEM-F12. Adherent colonies were scraped from the plate using a cell lifter, collected in a conical tube, and spun down at 1,000 rpm for 2 minutes. hPSC colonies were gently resuspended in hES media and plated on MEFs. The freshly passaged colonies were left untouched for one day before daily media changes resumed.

#### RBC Differentiation methods

hPSCs were directly differentiated into RBCs using three previously published protocols. The first method (Chang) differentiated hPSCs as embryoid bodies (EBs) for 14 days and then plated whole EBs on a matrigel membrane with hematopoietic cytokines to induce directed differentiation. It took 27 – 42 days to generate RBCs. Full details of the Chang differentiation method have been published [25]. The second method (Ma) differentiated whole hPSC colonies on murine fetal liver stromal cells for 15 days then transferred the cells to colony cultures and subsequently suspension cultures with hematopoietic cytokines for an additional 20 days to generate RBCs. Full details of the Ma differentiation method have been published [30]. The third method (Lanza) is more defined and serum-free. hPSCs were differentiated as EBs for 3.5 days then dissociated to single cells and plated in a methylcellulose based media with hematopoietic cytokines to promote hemangioblast expansion followed by erythroid differentiation and expansion. RBCs are generated after 21 days. The details of

the Lanza method have been published [29, 79], but we made the following 3 modifications for optimization. 1) hPSCs were passaged as described above. Passage with trypsin, as described in Lu *et al.*, led to loss of pluripotency. 2) EBs were dissociated with 0.25% trypsin for 2 minutes, flicking the tube every 30 seconds. After 2 minutes, EBs were triturated with a P1000 and passed through a 40  $\mu$ m strainer into a 50 mL conical tube, which was then filled with PBS + 0.1% BSA to dilute the trypsin. 3) No recombinant HoxB4 was used during the differentiation, as we achieved consistently high RBC yield without exogenous HoxB4.

#### Cord Blood Mononuclear Cell (CBMC) Isolation

Fresh cord blood was obtained from the Pasquarello Tissue Lab with approval from CHB. The cord blood was mixed with 4 volumes of cold PBS + 2 mM EDTA. Then, 35 mL was layered on top of 15 mL of Ficoll-Paque in 50 mL conical tubes and centrifuged at 400 x g for 30 minutes at 20°C. The mononuclear cell layer was collected and washed twice with PBS + 2 mM EDTA, centrifuging at 300 x g for 30 minutes at 20°C, followed by one last wash and centrifugation at 4°C.

#### MACS Enrichment

CBMCs were enriched for CD34<sup>+</sup> cells or CD71<sup>+</sup> cells using MACS (Miltenyi). CBMCs were stained with either 1) CD71-APC antibody or 2) CD34-APC antibody followed by anti-APC beads and resuspended in PBS + 2% serum, before flowing through an equilibrated LS column. All staining was for 15-30 minutes on ice. Percent enrichment was determined by flow cytometry.

#### FACS Analysis

Multicolor FACS analysis was carried out on a 5-laser-LSRII or LSRFortessa flow cytometer (Beckton-Dickinson), and sorting was performed on a FACS-Aria+UV (Beckton-Dickinson). Cells from the hemangioblast expansion phase were stained on ice for 15-30 minutes on ice with antibodies for

hematopoietic stem cells markers: CD34 – Pacific Blue, CD38 – PE, and propidium iodide. The cells were washed and resuspended in PBS + 2% serum. RBCs were stained on ice for 15-30 minutes on ice with antibodies to assess erythroid maturation: CD71 – APC, GPA – PE, Ep1 – FITC, and propidium iodide, and then either analyzed or sorted. Ep1 antibody was generously provided by Dr. Stamatoyannopoulos. For single cell qPCR, CD71<sup>+</sup> GPA<sup>+</sup> cells were sorted as single cells into 96-well plates containing 2  $\mu$ L lysis buffer (250 mM Guanidine Thiocyanate) per well.

To detect hPSC derivatives after transplantation, cells from the collected tissues were stained on ice for 15-30 minutes with antibodies for CD45 – PE/Cy5; APC/Cy7 (2 clones were used), CD71 – FITC, GPA – PE/Cy7, CD19 – PE, CD33 – APC, and DAPI. Cells were then washed and resuspended in PBS + 2% serum.

#### qPCR

RNA for bulk population qPCR was isolated from samples in Trizol and then reverse transcribed using the SuperScript II RT kit (Invitrogen). The resulting cDNA was used for qPCR with SYBR Green master mix and published primers for hemoglobins and erythroid transcription factors (Table 3-2).

cDNA was synthesized from single cells sorted into lysis buffer in 96-well plates using the SuperScript II RT kit reagents adapted for smaller reaction volumes. The cDNA from each well was then divided equally between 3 96-well plates for qPCR of  $\epsilon$ -globin,  $\gamma$ -globin, and  $\beta$ -globin using SYBR Green master mix.



Table 3-2: qPCR primers

Gene		Primer Sequence	Source
KLF11	forward	GCATGATAGTTTCTTGGGTCAACC	[80]
	reverse	GTAGGCGATGCTATAGTCCACAG	
EpoR	forward	TGGAAGAGGATGGAGGTCGG	[81]
	reverse	GCTGGGAAGAGTTGACCAACAG	
GATA-1	forward	GCCCTGACTTTTCCAGTACC	[82]
	reverse	CGAGTCTGAATACCATCCTTCC	
KLF2	forward	TGCGGCAAGACCTACACCAAGAGT	[83]
	reverse	AGCCGCAGCCGTCCCAGTT	
GATA-4	forward	AGAAAACGGAAGCCCAAGAAC	[84]
	reverse	CTGGAGTTGCTGGAAGCAC	
LMO2	forward	GGCGGCGCCTCTACTACA	[85]
	reverse	CTGGGCCGGAAGCTCTGCC	
SP1	forward	CAGACTCAGTATGTGACCAA	[86]
	reverse	GTTGAGTAGCTATTGGCATT	
SOX6	forward	GAGGCAGTTCTTTACTGTGG	[87]
	reverse	CCGCCATCTGTCTTCATAC	
SCL/Tal1	forward	GTTCTTTGGGGAGCCGGATG	[87]
	reverse	ACATTCTGCTGCCGCCATCG	
STAT5A	forward	TTACTGAAGATCAAGCTGGGG	[87]
	reverse	TCATTGTACAGAATGTGCCGG	
STAT5B	forward	CATTTTCCCATTGAGGTGCG	[87]
	reverse	GGGTGGCCTTAATGTTCTCC	
YY1	forward	AAGGCTGCACAAAGATGTTCA	PrimerBank
	reverse	CATAGGGCCTGTCTCCGGTAT	
FOG1	forward	CTGAAGAAGCCGCCAACTCA	[88]
	reverse	AAGGCGCACATATAGCAGTCC	
IKAROS	forward	CGGCTTTGTCTGGGAGTTG	[89]
	reverse	GCCCTTCTGGGTGAATGAG	
hBCL11A	forward	AACCCAGCACTTAAGCAAA	[61]
	reverse	GGAGGTCATGATCCCCTTCT	
hKLF1	forward	TTGCGGCAAGAGCTACACC	PrimerBank
	reverse	GTCAGAGCGCGAAAAAGCAC	
hMYB	forward	TACAATGCGTCGGAAGGTCG	[90]
	reverse	GCGGAGCCTGAGCAAAACC	

## CFU Assay

Cells from the hemangioblast expansion phase were collected with PBS + 0.1% BSA. Each CFU dish contained 25,000 to 50,000 cells mixed with 1.5 mL of MethoCult 4434. Colonies were counted after 14 days.

## Lentiviral Over-Expression of Transcription Factors in Differentiating hPSCs

BCL11A-L, BCL11A-XL, and KLF1 (GeneCopoeia) were cloned into lentiviral vectors using the Gateway system. Genes of interest were first recombined into pENTR, then shuttled to either pSMAL or pLenti. The GFP control plasmids were purchased directly (GeneCopoeia). 293T cells were transfected with plasmids for the genes of interest along with third-generation packaging plasmids. Virus was harvested 24 hours after transfection and concentrated by ultracentrifugation at 22,000 rpm for 2.3 hours. Viral titers were determined by serial dilution on 293T cells.

During the entire viral infection process, hPSC derivatives were kept in media similar to that of step 2 media from the Lanza protocol, except that StemLine I media replaced the methylcellulose. After EB dissociation, cells were infected with concentrated virus (10 MOI) by centrifuging the cells with virus and protamine sulfate for 30 minutes at 2,000 rpm. The virus was left on the cells for 24 hours, then washed off with PBS + 0.1% BSA. Cells were plated in regular step 2 media containing methylcellulose and differentiation continued as previously described. The efficiency of viral infection was determined by flow cytometry after 72 - 96 hours.

## Co-culture During Differentiation

Dissociated EBs in normal step 2 media were seeded on top of 70,000 human bone marrow (HS-5) and fetal liver (FL62891) stromal lines (ATCC). The differentiating cells remained in co-culture for the remainder of the differentiation.

## Animals

NSG and NSG-SGM3 mice were purchased from Jackson Laboratories and bred in house (strains 005557 and 013062). Experiments were carried out with IACUC approval from CHB.

## Transplantation Assay

NSG and NSG-SGM3 neonates, 1-2 days old, were conditioned with 100 rads of radiation. Neonates received  $1 \times 10^6$  cells from day 6 or day 8 of step 2 resuspended in PBS + 0.1% BSA and injected either intravenously in 15  $\mu$ L or intrahepatically in 50  $\mu$ L. Recipients were euthanized at 2, 4, or 6 weeks post-transplant for collection of the thymus, spleen, liver, lung, bone marrow, and peripheral blood. Peripheral blood and bone marrow were subjected to red blood cell lysis for 20-30 minutes on ice. Solid tissues were mashed over a 70  $\mu$ m filter for flow cytometry.

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## **Chapter 4**

### **Perspective**

## 4.1 Research Summary

This thesis furthers our understanding of the developmental maturation of hematopoietic cells, specifically hematopoietic stem cells (HSCs) and red blood cells (RBCs), to the definitive fate. We found that the environment both *in vivo* and *in vitro* has a significant effect on the emergence and function of definitive cells. In the context of HSC transplants, the hematopoietic environment of the transplant host significantly affects the outcome of the transplant with HSCs from different developmental stages. In the context of directly differentiating human pluripotent stem cells (hPSCs) into RBCs, the culture environment may lack the cues necessary for the emergence of definitive RBCs.

HSC transplants using adult and neonatal recipients are established systems we used to highlight functional differences between early embryonic and adult-like HSCs. We quantified the number of HSCs in the early embryo with limiting dilution transplants of neonatal recipients. When unfractionated early embryonic tissues were transplanted into neonates, our analysis predicted 1 repopulating cell in the para-aortic splanchnopleura/aorta-gonad-mesonephros (PSP/AGM) of 44.8 E9.5 embryos, 2.84 E10.5 embryos, and 0.96 E11.5 embryos. Interestingly, the neonatal host is not more permissive for engraftment of all HSCs. Adult-like HSCs, even those from the embryo, preferentially engrafted adult recipients. From limiting dilution transplants, the predicted frequency of HSCs in the Lineage<sup>-</sup> Sca1<sup>+</sup> cKit<sup>+</sup> CD150<sup>+</sup> CD48<sup>-</sup> compartment of E14.5 fetal liver and adult bone marrow was 4.4- to 13.5-fold lower, respectively, with neonatal recipients compared to adult recipients. We noticed HSCs from the fetal liver engrafted neonates better than HSCs from adult bone marrow. Further evaluation of HSCs residing in liver versus bone marrow demonstrated reduced neonatal engraftment from HSCs that had migrate to the bone marrow by the time of birth.

We investigated potential mechanisms governing the reduced neonatal engraftment potential of adult HSCs. We showed that adult HSCs exposed to interferon- $\alpha$ , thus forced to enter the cell cycle, engrafted neonates better than controls, suggesting proliferation of adult-like HSCs enhances neonatal engraftment potential. We also found that adult HSCs homed mostly to the liver in neonatal recipients and to the liver and spleen in adult recipients, which is likely linked to the observed difference in long-term engraftment potential in the two transplant models.

Our findings have implications in functionally testing hematopoietic derivatives of human pluripotent stem cells (hPSCs), as hematopoietic derivatives of hPSCs are generally embryonic [1-3]. We evaluated the potential of hPSC derived RBCs to transition to the definitive fate.

We compared the RBC output of 3 different directed differentiation methods with 11 hPSC lines and optimized the most robust and reproducible combination, the Lanza protocol with MSC-iPS1 cells [4]. hPSC derived RBCs are primitive with high levels of  $\epsilon$ -globin and  $\gamma$ -globin and negligible amounts of  $\beta$ -globin. We attempted to promote a more developmentally mature definitive state by over-expressing key erythroid transcription factors BCL11A and KLF1, co-culturing on human fetal liver and bone marrow stromal cells, and providing a hypothesized supportive *in vivo* environment by transplantation into neonatal immunodeficient mice producing 3 human cytokines. Regardless of the approach used, we did not detect a significant or consistent change in the ratio of expressed hemoglobins; therefore, we concluded the fate of the cells was not altered. Our results highlight the significant role of the environment in properly maturing hematopoietic cells.

## 4.2 Discussion

Current methods of differentiating PSCs yield embryonic cells instead of the largely desired adult definitive cells [1, 5-7]. We set out to gain insights into the transition to the adult definitive fate, choosing two systems, HSCs and RBCs, with well characterized *in vivo* counterparts and markers distinguishing the embryonic from the definitive cells [8, 9].

The gold standard functional measure of definitive HSCs is long-term multi-lineage reconstitution upon transplantation into a lethally irradiated wild-type adult mouse, as it most closely mimics clinical cases [10-13]. However, this assay may not accurately capture all types of HSCs. Imaging studies suggest the presence of HSCs in the early embryonic tissues at E10.5 and earlier, which have low or no engraftment in wild-type adult recipients [14-17]. Interestingly, the PSp/AGM and yolk sac from E9.0 to E10.5 engraft immunodeficient adult mice and wild-type neonatal mice suggesting that the proper supportive environment is necessary for HSCs to contribute to long-term multi-lineage hematopoiesis [18-20].

Reports from Yoder and colleagues suggested neonatal recipients are permissive for engraftment of early embryonic HSCs [19, 20]. We used the neonatal transplant model to quantify the number of HSCs present in the PSp/AGM from E9.5 to E11.5. Our quantification data indicate the presence of rare intraembryonic HSCs in approximately 2% of E9.5 embryos. The frequency of embryos with HSCs increases as the embryos continue to develop. At E11.5 there is at least 1 HSC per embryo, as predicted by LDA using neonatal and adult recipients [21]. This quantification paired with imaging studies furthers our understanding of the emergence of the earliest definitive HSCs, suggesting that HSCs continuously emerge in clusters budding from the ventral floor of the PSp/AGM from E9.5 to E12.

The emerging clusters may contain pre-HSCs that are still maturing in response to the embryonic environment [22]. The neonatal environment, unlike the adult environment, may pattern pre-HSCs such that they are able to contribute to long-term multi-lineage engraftment, but there is a bias towards the B-cell lineage, suggesting that the pre-HSC could be related to a described B-cell progenitor that emerges directly from the hemogenic endothelium at E9.0 [23]. The B-cell skewing is reduced over two days as the pre-HSCs mature into HSCs capable of reconstituting wild-type adult recipients by E11.5.

One source of variability in the LDA is the conditioning regimen. We made the conditioning regimen of neonates and adults as similar as possible, giving both sub-lethal irradiation with no helper cells or the highest tolerated irradiation dose with helper cells. The number of helper cells used as short-term radioprotection in lethally irradiated adult recipients significantly affects engraftment results and therefore quantification of HSCs. Medvinsky's group reported that a low dose of 20,000 helper cells contains an average of 2 HSCs, which reduces competition with the small number of donor HSCs for space in the niche [21, 24]. In our adult transplants, lowering the helper dose 15-fold to 20,000 cells resulted in a 10.6-fold increase in the number of predicted HSCs. Transplanting neonates with helper cells resulted in similar or slightly higher engraftment than we observed in neonates that did not receive helper cells. The irradiation dose also affects transplant outcomes. The sub-lethal neonatal conditioning may even result in an underestimation of the HSC frequency as there is less space cleared in the marrow and more competition from remaining endogenous cells. Adult recipients conditioned like neonates with sub-lethal irradiation and no helper cells had slightly improved donor chimerism, but the neonatal system still proved more permissive for engraftment of HSCs from E10.5 and earlier. Although we did not examine the effects of altered conditioning regimens in transplants with adult-like HSCs, the substantial disparity between neonatal and adult recipients is likely a result of variations in the conditioning regimen and additional elements such as different hematopoietic niches or different growth factors.

In addition to quantifying the HSCs present in the PSp/AGM of the early embryo, we demonstrated that neonatal recipients are not more permissive hosts for adult-like HSCs. As the current gold standard in the field is to define HSCs by transplantation into wild-type adult recipients, our findings are critically important, showing the effect of the recipient on the outcome of a transplant. Future studies will identify properties of HSCs that preferentially engraft neonates over adults and vice versa, which may lead to the discovery of novel hematopoietic stem/progenitor populations in more mature tissues like the adult bone marrow.

Our findings exemplify the selective pressure on donor cells that is created by the transplant environment, which significantly affects transplant outcomes and therefore our conclusions about HSC biology. To make correct conclusions from functional HSC transplant studies, we must take into account the survival and proliferation needs of donor HSCs and perform transplants with the proper host to address the specific experimental question.

It is very likely that different niches support HSCs in neonates and adults. While the complexity of the adult bone marrow niche is coming into focus, the HSC niches in neonatal liver and bone marrow are not well studied [25, 26]. We found that LT-HSCs from the neonatal liver engrafted neonates more robustly than LT-HSCs from the neonatal bone marrow, suggesting functional variation in neonatal HSCs. We also showed that adult HSCs home to different niches in neonatal and adult recipients, suggesting the presence of different hematopoietic niches. Future studies of the neonatal HSC niche will shed light on the differing needs of early embryonic and adult HSCs. A more exhaustive homing study of short-term time points would provide insight as to the hematopoietic tissue in neonates and adults that supports long-term engraftment from HSCs taken from different developmental stages.

The presence of a niche that supports the transplanted HSC is critical for long-term engraftment. Frenette and colleagues recently identified unique niches within the adult bone marrow for proliferating

and quiescent HSCs [26]. Coupled with our data suggesting cycling adult-like HSCs have enhanced neonatal engraftment potential, it is likely that neonatal bone marrow contains more niche sites supporting proliferating HSCs than quiescent HSCs. Alternatively, donor HSCs may need to be cycling to compete with remaining endogenous HSCs in the sub-lethally irradiated neonates. Concentrations of steel factor in neonatal hematopoietic niches may also play a role in the neonatal engraftment potential of adult-like HSCs [27].

Our work highlights the important role the host environment plays in HSC transplants. In functionally testing hematopoietic derivatives of hPSCs, it is also necessary to keep the role of the environment in mind. To date, hematopoietic derivatives of hPSCs are embryonic [1-3]. Our work with hPSC derived RBCs suggests the *in vitro* environment may also play a substantial role in the survival and differentiation potential of definitive HSCs.

We tested 11 hPSC lines in 3 published RBC differentiation protocols and found one robust and reproducible combination, MSC-iPS1 cells in the Lanza protocol [4]. All hPSC derived RBCs were primitive, containing mostly  $\epsilon$ -globin and  $\gamma$ -globin with very little, if any,  $\beta$ -globin. The ratio of expressed hemoglobins did not change with co-culture on fetal liver or bone marrow stromal cells or with over-expression of BCL11A or KLF1. Single cell analysis did show 1 cell out of 44 with high-levels of  $\beta$ -globin mRNA, suggesting the rare emergence or survival of definitive RBCs. Our data suggest the *in vitro* transition to the definitive fate is a rare occurrence. There are many possible explanations including: 1) the derived RBCs are too primitive to respond to our methods, 2) there are two distinct waves of erythropoiesis and additional or different factors are needed to convert from the primitive progenitor to the definitive progenitor, or 3) current culture conditions do not support the emergence of definitive cells. These hypotheses can be tested by manipulating more mature cells expressing mostly  $\gamma$ -globin



[28] or using our optimized system to screen for additional factors that facilitate the transition to the definitive fate.

Additionally, our data demonstrate primitive RBCs and primitive RBC precursors are unable to thrive in neonatal xenografts. In control transplants with CD34<sup>+</sup> enriched cord blood cells, we observed engraftment at lower levels than expected from adult recipients, which may be related to our observations with adult-like HSCs in congenic murine transplants. Several potential scenarios could explain the lack of engraftment from hPSCs derivatives: 1) transplanted cells may not survive *in vivo*, 2) the cells cannot home to the proper niche, 3) surviving cells may not proliferate and thus are too few to detect at analyzed time points, and 4) an engrafting cell may not have been generated *in vitro*. Some of these hypotheses can be tested with short-term homing studies. A homing defect is likely not the problem as we did not detect engraftment when cells were injected intrahepatically.

If a definitive HSC did emerge *in vitro*, it is unlikely to have survived. Engineering the components of an environment supportive of self-renewing definitive HSCs that maintain the potential for multi-lineage differentiation is a challenge in the HSC field [29]. Thus, it is not surprising that we are unable to generate lineage committed cells of the definitive fate. Current *in vitro* cultures may promote stress erythropoiesis which up-regulates  $\gamma$ -globin [30-32]. There are two possible explanations for this: 1) the primitive and definitive precursors are extremely plastic, easily transitioning to the fate best supported in a particular environment, or 2) the primitive and definitive precursors have limited plasticity to transition from one fate to the other. With culture conditions supporting the emergence of the primitive fate, there would be no chance of a definitive cell emerging even if stimulated with transcription factors.

From the murine model system, we know there are two waves of hematopoiesis, and the primitive and definitive waves respond to different environmental stimuli [8, 33, 34]. Analysis of hPSC

derived RBCs suggests they are similar to RBCs emerging during yolk sac erythropoiesis, which may be too primitive to respond to the added transcription factors or co-cultures [8, 35]. Given that unique conditions give rise to primitive and definitive murine hematopoiesis [36-40], we may need an entirely different approach to hPSC differentiation to create the proper environment for the rapid emergence of definitive precursors. Alternatives include the carefully crafted differentiation protocol from Keller's group that slowly steers hPSCs towards the definitive hematopoietic fate [41]. The cultures go through an early primitive wave then begin to produce potential definitive precursors. The differentiation may be more efficient if the niche was recreated *in vitro*, promoting developmental maturation through cell-cell interactions and secreted factors. Recent reports implicate the Lin28-let-7 axis as a master regulator of developmental timing [42, 43]. Modulating the Lin28-let-7 axis during hPSC differentiation may provide the molecular signal necessary for the emergence of definitive hematopoietic cells. It is also feasible that the source of difficulty is the starting hPSCs.  $\beta$ -globin expressing RBCs have been derived from mouse PSCs [31, 44], suggesting the degree of pluripotency is the key as mouse PSCs are more naïve than hPSCs [45, 46]. More naïve hPSCs, like hLR5 cells, may better differentiate into definitive RBCs [47]. Once we understand the process of developmental maturation to definitive hematopoietic cells, we should be able to engineer methods to bypass the primitive wave entirely to rapidly produce definitive cells.

#### 4.3 Future Directions

Our work demonstrates the impact of the environment or culture conditions on the fate of hematopoietic cells. In the context of murine HSC transplants, HSCs from different stages of ontogeny have a preference for different recipients. There are unanswered questions concerning the differing

survival and differentiation needs of HSCs from different stages of development as well as questions surrounding different HSC niches and host environments in neonatal and adult recipients. We focused on the properties of adult-like HSCs linked to different recipient preferences, but there is more to be learned. We showed that adult HSCs exposed to interferon- $\alpha$  had enhanced neonatal engraftment potential, but we did not see the same result with adult HSCs exposed to 5-FU, suggesting the interferon- $\alpha$  effect may be more complex than simply increasing proliferation. Additionally, we demonstrated that adult-like HSCs have reduced neonatal engraftment potential once they migrate from the liver to the bone marrow. It has yet to be determined if this is caused by the bone marrow microenvironment, perhaps related to the expansion of  $\alpha$ -HSCs, or if there are cell intrinsic changes reducing neonatal engraftment potential that coincide with migration to the bone marrow.

There is also the question of the mechanism causing early HSCs to preferentially engraft neonatal recipients. Many avenues of investigation could provide insights. The neonatal preference may be an immunologic phenomenon, which could be studied by closely examining MHC markers on early embryonic HSCs versus adult-like HSCs or by transplanting neonatal mice from an immunodeficient strain [48]. There are obviously differences in cytokines and growth factors between neonatal and adult recipients. Neonates have lower cytokine levels that may not be sufficient to invoke a response from adult HSCs, which thrive in an environment with high cytokine levels [49]. Also, which niche and tissue early embryonic HSCs home to in neonatal and adult recipients is unknown. Studies in that vein would provide important information about the environment that supports the survival, maturation, and differentiation of the earliest HSCs, which may also provide insights into the niche that gives rise to the earliest HSCs in the embryo in addition to the proper culture environment to generate definitive hematopoietic cells *in vitro*.

Our work has implications for HSC transplants in the clinic and functionally assaying *in vitro* derived HSCs. In the clinic, donor cells for HSC transplant commonly come from umbilical cord blood, adult bone marrow, or adult mobilized peripheral blood [50-52]. To see if our findings translate to the human hematopoietic system, clinical data could be retrospectively analyzed correlating the source of donor cells, the age of the recipient, and engraftment outcomes. It would also be interesting to see if embryonic cells from other hematopoietic lineages, specifically RBCs, prefer neonatal hosts for clinically relevant procedures like transfusions.

In the time since this body of work was started, new differentiation methods have been developed to derive RBCs from hPSCs. Of particular interest is the work from the Orkin group that generated RBCs expressing only  $\gamma$ -globin [28]. It would be interesting to see if those cells or their precursors, which are more definitive than the cells we analyzed, could be forced to complete the transition to the adult definitive fate and express  $\beta$ -globin. To reach the adult definitive state, the chromatin may need to be made more accessible by adding epigenetic modifiers during differentiation [53, 54].

We highlighted the role of the environment and culture conditions on the self-renewal and differentiation capabilities of hematopoietic stem/progenitor cells, but there is much left to discover in the mechanisms governing the observed phenomenon. A complete understanding of the transition from the primitive fate to the definitive fate will not only teach us about a basic biological switch but will also give us the knowledge to mimic nature to generate cells for *in vitro* disease modeling, drug screening, and cell therapies.

#### 4.4 References

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